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Mechanisms Responsible for the Origin and Distribution of Blood-borne Tumor Metastases: *A Review**

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Two distinguishing biological characteristics of malignant tumors are their ability to invade adjacent normal tissues and their ability to produce secondary tumors in distant parts of the body. Local tissue invasion is a prerequisite to the formation of blood-borne metastases, for thus the tumor cells gain entrance to the vascular system. Therefore, an understanding of the mechanisms of metastasis begins with inquiry into the phenomenon of invasiveness. It is the purpose of this review to re-examine our concepts concerning these two features of neoplasia in the light of knowledge gained from experiments during the past ten or more years. The need for such a review is evident from the usual textbook treatment of the subject; the concepts presented therein are essentially those advanced by the earlier pathologists as a result of their interpretations of autopsy material. This has become misleading, for there has accumulated an important body of knowledge, derived from carefully conducted experiments, that adds considerably to our understanding of these fundamental processes. The present review is not intended as an exhaustive treatise including every experimental approach to these problems; rather, it is an attempt to sift out what appears, at this time, to be most pertinent and to arrive at tentative concepts that seem to account for the major part of the observed events.

MECHANISMS OF INVASIVENESS

By invasiveness is meant the ability of cells to penetrate the tissues surrounding them. Invasiveness is not restricted to the cells of malignant tumors. Some of the normal cells of the body—leukocytes and macrophages, for example—invade other tissues as readily as do cancer cells. Thus, the capacity to permeate other tissues is a quality peculiar to certain cells, normal or malignant. Cells of malignant tumors derived from noninvasive normal cells, therefore, must have some biological attribute not shared by the cells of benign tumors or by most normal cells, but present in leukocytes and macrophages. As Willis said, ". . . the invasive properties of tumors reside largely or entirely in the tumor cells themselves" (53).

Such factors as multiplication rate, liberation of lytic substances, and loss of growth restraints can no longer be regarded as essential factors in invasiveness, since these qualities either are shared by noninvasive tumors or do not exist in malignant tumors.

The invasive properties of leukocytes and macrophages depend on their being isolated single cells and on their highly developed ameboid motility. That cancer cells also possess ameboid motility was reported as long ago as 1863 by Virchow (48) and has been demonstrated in tissue culture by many workers in recent times (8, 11, 18, 23, 26, 31, 33, 35, 43). These tissue culture studies have shown beyond all doubt that tumor cells in general are ameboid, whether they are of mesodermal or epithelial origin.

Neoplastic epithelial cells from carcinomas of man and of laboratory animals have been photographed with a moving picture camera and their rates of speed determined (23). Cells from breast cancers, for example, moved at an average of 0.7 μ/min, with a maximum rate of 2.4 μ/min. Cells from a carcinoma of the kidney attained

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a maximum rate of 4.4 μ/min, while some cells from a mouse fibrosarcoma traveled at 6.2 μ/min. Of additional interest is the observation that even tiny clusters of from three to five epithelial cells from a rabbit carcinoma progressed as an ameboid unit. It was also demonstrated that the cells of benign epithelial tumors and even non-neoplastic glandular epithelial cells (from cystic disease of the breast) may be motile in tissue culture, *provided that these cells are first forcibly detached from their companion cells.* It appears probable, then, that the local invasiveness of cancer cells depends upon their ability to progress by ameboid movement.

Why, then, do not the cells of benign tumors or the cellular components of the various organs also invade, since they too, at least in some instances, are capable of ameboid motility? There is evidence to indicate that the cells of normal tissues and also the cells of benign tumors are so firmly attached to one another that they are unable to escape. In contrast, the cells of malignant tumors are often found in the body free from the parent tumor.

The reason that the cancer cells become free is their greatly reduced adhesiveness. Micromanipulation studies (12) have shown that the magnitude of force required to separate a pair of normal squamous epithelial cells from each other is much greater than the force required to separate cancerous squamous cells. Also, the individual cells from various malignant adenocarcinomas from man were found to be easily dislodged by a mechanical shaking of the cancerous tissue, whereas shaking the normal prototype tissue dislodged few or no cells (37).

The deficiency in the adhesiveness of cancer cells, then, permits them to separate easily from their mutual attachments. Once free, they are capable of ameboid motility.

The lowered adhesiveness of cancer cells has been related to a deficiency in calcium (12, 20). Recognition of the role played by calcium in maintaining the adhesiveness of normal cells dates back to the observations of Herbst (27) on the blastomeres of sea-urchin eggs. When placed in a calcium-free medium, the individual blastomeres become separated, losing their ability to cling to each other. Calcium deficiency in cancerous tissues has been reported by several independent investigators (4, 7, 10, 20, 21). For example, the calcium content of a group of human cancers has been found, by flame spectrophotometric determinations, to be about 40 per cent less than that of the normal prototype tissues. That the loss of calcium in the cells is dependent upon their neoplastic character, rather than upon the fact that such cells are rapidly multiplying, is emphasized by observations made upon regenerating rat livers. In this material, although the cells were actively proliferating, there was no demonstrable decrease in calcium (20). Hepatomas of rats, on the other hand, are deficient in calcium (25). Moreover, procedures designed to decrease the calcium content of normal cells have resulted in reducing their adhesiveness. It was shown, by Carruthers and Suntzeff, that methylcholanthrene, when applied to mouse skin, causes a reduction in the calcium (9, 47), and, by Zeidman, that this same substance causes a loss of adhesiveness when applied to normal squamous cells (55).

The possibility that cancers liberate hyaluronidase and that this enzyme, by hydrolyzing the hyaluronic acid of connective tissues, opens pathways for the invading cancer cells has been explored (5, 6, 16, 22, 36). That hyaluronidase may somewhat increase the invasiveness of tumors that are invasive to start with has been reported by Simpson (45). When cancers do contain hyaluronidase, the enzyme may possibly facilitate invasion of the surrounding normal tissues, but that it is a requisite to, or even a frequent factor in, invasiveness appears doubtful.

From the combined evidence it seems safe to conclude that the invasiveness of cancer cells depends largely upon a loss of adhesiveness that is associated with, if not due to, local calcium deficiency. Most cell physiologists agree that calcium is located principally at the cell surface, but how it is combined there and why the cancer cell is unable to bind the normal amount of calcium is as yet unknown. Reduced adhesiveness, however, is what permits the cells to become free-living, detached units that thereafter progress by their own ameboid motility.

THE RELATION OF INVASIVENESS TO METASTASIS

As described earlier, it is characteristic of cancer cells to separate from one another and then to travel by ameboid motion through the tissues. In their course of travel they follow paths of least resistance. They penetrate loose tissue such as muscle or areolar tissue more readily than hard and compact tissue such as bone and cartilage, the latter often remaining long intact. Cancers of the prostate, for example, infiltrate the loose tissues of the nerve sheaths in preference to the dense parts of the gland itself (29, 38, 52). The *lymphatics* offer natural preformed paths of low resistance. Along these avenues cancer cells may grow as solid cords or be carried along in the current. Similarly, cancer cells in general have little difficulty in penetrating

the walls of capillaries and veins, whereas invasion through the tough muscular wall of an artery is rarely accomplished (53, p. 160).

Penetration of *veins* and *capillaries* places the cancer cells in position to be swept away by the currents of circulating blood and so carried to distant parts of the body. Single cells or small clumps of cells become detached from their point of entrance to the vessel and are carried along by the blood stream. At times, however, the invading cells may multiply locally to form solid cords of tumor within the vessels, even large veins becoming packed with masses of neoplastic tissue. In this way tumor may extend within the vessel as a continuous cord for considerable distances.

Local invasiveness, then, places the cancer cells within the vascular channels through which they may be transported to distant sites. Lodgement followed by multiplication of the cells in the new site produces a metastatic tumor.

MECHANISMS OF BLOOD-BORNE METASTASIS

This review is limited to consideration of metastasis by way of the blood stream. Metastasis by implantation on serosal and mucosal surfaces has been omitted because of the dearth of experimental data in this field. This is also true of metastasis via the lymphatics, though Zeidman has made preliminary studies[1] and further work should correct the deficiency in knowledge about the spread of tumors through the lymph channels. Observations on autopsy material have been reported in detail by others (50, 53, 54).

The two major specific questions about metastasis that have concerned students of cancer are:

1. What factors determine the *number* of metastatic tumors produced by the primary growth?
2. What factors determine the *anatomical distribution* of metastatic tumors?

The answers to these questions depend in turn upon the following fundamental processes:

1. *Lodgement of the embolic tumor cells.*—In what organs, and in what parts of the vascular system within the organs, are the tumor cells arrested, and does this differ from organ to organ?
2. *Survival of the arrested embolic cells.*—Are the cells as capable of survival in one part as in another, or do peculiarities in the local environment affect their survival, favorably or adversely?
3. *Establishment of a new blood supply and stroma.*—Are there differences in the ability of normal tissues to furnish the lodged tumor cells with supporting connective tissue and blood vessels?
4. *Growth of the tumor.*—Are there local environmental conditions in different parts of the body that affect, favorably or adversely, the rate of multiplication of the tumor cells?

[1] I. Zeidman, personal communication.

FACTORS AFFECTING THE NUMBER OF METASTATIC TUMORS

What factors determine the *number* of metastases produced by a primary tumor?

As might be expected, the *number of embolic cells* is an important factor. This was demonstrated by injecting different numbers of cells of a transplantable sarcoma into the tail vein, in mice, and counting the number of resulting tumors in the lungs. A direct proportionality was found between the number of living embolic cells and the number of tumors (57).

The mortality of embolic cells is very high. Warren and Gates (51) studied the fate of tumor cells introduced into the systemic veins of the mouse. Only a small percentage survived. The enormous majority of embolic cells fail to establish themselves in the lungs and to form tumors (28, 57). These findings confirm the conclusions drawn from study of autopsy specimens (44, 53, pp. 175, 179).

Since, as stated above, the number of tumors is proportional to the number of embolic cells, the original question concerning the number of metastases produced by a primary growth may be restated as follows: What factors in the primary tumor affect the number of tumor cell emboli released into the circulation?

The age of the primary tumor.—With the use of a transplantable tumor in inbred mice, the number of metastases was found to be proportional to the duration of growth of the primary tumor (57). Animals were sacrificed at different times after tumor implantation, and metastatic lung tumors increased in numbers with the age of the primary growth. That is, the longer the tumor existed, the greater were the number of metastases.

The size of the primary tumor.—It is a well established fact that the size of the primary tumor in man is no criterion of the number of metastatic tumors. In man, it should be emphasized, both host and tumor constitute uncontrollable variables. However, under experimental conditions, these factors can be controlled. In inbred strains of mice, the identical tumor may be implanted in almost identical hosts. Under these conditions, it might be expected that the size of the primary tumor would have an appreciable effect on the number of metastases, since the number of potential embolic cells would be greater in large tumors than in small ones.

When mice were inoculated with fragments of a transplantable tumor, those that received larger

implants developed more metastases than those receiving smaller ones (57). However, when the *final* sizes of implanted tumors were compared to the number of metastases, no correlation was found, as the number of metastases varied so greatly from one animal to another. This result is consistent with what is found in man: the number of metastases is not apparently related to the final size of the primary tumor. In the study just cited, the wide scatter in the number of secondary tumors, even under these controlled conditions, indicates the existence of factors as yet unknown that influence the number of metastases far more effectively than does the element of size.

Factors Affecting the Distribution of Metastatic Tumors

The distribution of metastatic tumors is a problem that has fascinated pathologists and clinicians for many years, but this problem has been stared at rather than investigated. Why are some organs frequently the site of secondary tumors, while others are rarely so? Why do some tumors metastasize frequently to one organ and only rarely to another? By what pathways do tumor cells travel from a primary growth to some remote portion of the body?

It was in an attempt to answer these questions that Paget (39) likened embolic cancer cells to seeds scattered in soils of different degrees of fertility, to grow only where the soil was suitable. This "soil" hypothesis was based upon the supposition that local chemical factors rendered some parts of the body (e.g., muscle and spleen) relatively unsuitable for the development of secondary tumors, while in other organs (e.g., bone marrow and liver) conditions were specifically favorable for the survival and multiplication of embolic tumor cells. The "soil" hypothesis has found wide acceptance.

An alternative hypothesis, a mechanical explanation, was proposed by James Ewing, who stated: "The mechanics of the circulation will doubtless explain most of these peculiarities, for there is as yet no evidence that any one parenchymatous organ is more adapted than others to the growth of embolic tumor cells" (24). This sentence was deleted from the final edition of Dr. Ewing's book, suggesting that he became less certain of its tenability.

Metastatis through Venous Channels

The three commonest sites of metastatic tumors in man are the lung, the liver, and the bones of the axis and trunk (1, 17, 30, 32, 42, 49, 53, p. 178, 54). That the lungs and liver should be so frequently involved is readily understandable, since the lung receives the venous drainage from the caval system, and the liver the portal drainage. But why the bones of the axis and trunk? The "soil" hypothesis would account for the frequency of secondary tumors in the bones generally by attributing to them peculiarly favorable conditions for tumor development. Presumably, according to this concept, the pelvic bones and shoulder girdle are better "soil" than are the tibia and radius! However, an alternative explanation is available.

Oscar Batson (2, 3) suggested that tumor cell emboli from the prostate might enter the vertebral venous system and be carried directly to the bones of the spine, pelvis, and skull, by-passing the lungs entirely. He showed that increase in intra-abdominal pressure, such as would result from coughing or straining, would facilitate the passage of embolic cells into the vertebral veins by diverting the flow of blood from the caval system into the vertebral veins. Recently, it has been shown by experiment (13) that this pathway is indeed used. Suspensions of tumor cells injected into the femoral veins of rats and rabbits, while the abdominal pressure was slightly elevated, were diverted into the vertebral veins and produced tumors in the vertebrae. Tumor cell emboli were found plugging the ramifications of the vertebral veins.

Since the vertebral veins form an extensive system from the pelvis to the skull, anastomosing freely with the caval system at each segmental level, yet not subject to pressure changes within the body cavities, it is not surprising that embolic cells readily enter these vessels and so lodge in the spine, skull, and pelvis. The mammary veins, for example, communicate with the vertebral system through the intercostal and subclavian veins; hence it is understandable that in man breast cancer should metastasize frequently to the spine and pelvis. The thyroid occupies a position in the neck as favorable for this route as that of the prostate in the pelvis. Indeed, since the vertebral and systemic veins communicate at each segmental level, it is not surprising that tumors in almost any location may sometimes metastasize to the axial bones.

It would appear, then, that the three most common foci for metastatic tumors—the lungs, the liver, and the bones of the skeletal axis—are situated as one would expect them to be, on the basis of the anatomical arrangement of the veins of the body.

Metastasis through Arterial Channels

Less commonly, metastatic growths appear in other areas, such as the kidneys, adrenals, spleen, and muscle, emboli having reached these organs

through arteries. In these instances the embolic tumor cells must have gained entrance to the arterial side of the circulation by filtering through the vascular bed of the lungs (this possibility will be discussed later), or by secondary metastasis from pulmonary foci (50). Also, the liver and lungs may receive emboli through arteries (the hepatic and bronchial, respectively) instead of through the veins, as is more frequent. The bones, too, may receive emboli through the arterial circulation (50). This is true not only of the bones of the axis and trunk but of all the bones in the body.

Having entered the arterial blood stream, the cells may be carried anywhere. Why, then, of those organs receiving their emboli exclusively by the arterial circulation, are some more frequently the site of metastases (kidney and adrenal) than others (muscle and spleen)?

Recent work designed to answer these particular questions will now be described in some detail.

By good fortune there is available a transplantable tumor of rabbits, the V_2 carcinoma, that has the property of spontaneously metastasizing to the lungs and regional lymph nodes but, except in rare instances, to no other organ. Here, to all appearances, is a tumor the behavior of which conforms to the "soil" rather than the mechanical hypothesis. From the mechanical standpoint, the explanation for this behavior is that tumor emboli in adequate numbers rarely reach organs other than lungs and regional lymph nodes, whereas, if they could be made to reach other organs, tumors would appear there.

This prediction has been verified by experiment. Cell suspensions of the V_2 carcinoma were injected into the left side of the heart, which meant that, necessarily, emboli would be carried to every organ of the body. Accordingly, tumors appeared and grew in virtually every organ (15). Thus, it became evident that the V_2 carcinoma fails to metastasize widely, not because the various organs offer an unfavorable chemical environment but because adequate numbers of tumor cells do not reach those organs.

Still left entirely unexplained, however, was the fact that metastatic tumors were far more numerous in some organs than in others—as, of course, is true in spontaneous metastases of every kind of tumor. Thus, in the experiments just cited, tumors were numerous in kidneys, eyes, adrenals, but infrequent in spleen, muscles, and thyroid. The mechanical hypothesis had to account for these differences if it were not to be invalidated.

The "soil" concept would explain the unequal distribution of the V_2 carcinoma as owing to (*a*), the inability of all but a few tumor emboli to establish a blood supply in such unfavorable soil as muscle and spleen or (*b*) the fact that, even if some emboli survived in these organs, metastatic tumors would grow very slowly because of unfavorable chemical conditions and might not even attain macroscopic dimensions.

These explanations, deduced from the "soil" hypothesis, were tested in rabbits, rats, and mice with four different kinds of tumors (19). Fragments of these tumors were implanted into muscle, spleen, liver, kidney, and adrenal. There was no difference in the number of "takes" in the several organs; there was no observable difference in the rate of growth, except that one type of rabbit tumor grew slightly faster in the spleen than in the other organs tested. Therefore, these experiments did not support the view that tumors acquire a blood supply more easily, and subsequently grow better, in some organs than in others.

Another explanation for differences in organ-distribution of tumors was then tested (14). According to this explanation, the reason that greater numbers of secondary tumors are found in the kidney and adrenals than in the spleen, muscles, and thyroid is that greater numbers of tumor emboli reach the kidney and adrenal than the other organs; the number of tumors in each organ was predicted to be proportional to the number of emboli that reach it.

To ascertain the relative number of tumor emboli reaching the several organs, cells of the Brown-Pearce rabbit tumor were fixed and stained, to facilitate recognition, and then injected into the left side of the heart. Animals were sacrificed at once, histological sections were prepared, and the emboli in arterioles and capillaries were counted, and computed as emboli per square centimeter, for each of the eight organs. Living tumor cells were similarly injected, in another series of animals, which were allowed to survive until the tumors had time to grow. Then the tumors were counted in histologic sections.

When the total number of stained cells reaching an organ was compared to the number of tumors in that organ, correspondence was only fair. However, if only those embolic cells that lodged in the capillaries were considered, correspondence was excellent. Thus, the greatest number of emboli per square centimeter was found in iris, pituitary, adrenals, and kidneys; the smallest number in muscle, thyroid, and spleen. The same frequency distribution was found for the tumors.

It was further demonstrated that the anatomical frequency distribution of the *naturally* occurring metastases of this tumor was predictable on the basis of the distribution of the embolic stained

cells; that is, the agreement between the lodgement of stained cells was almost exactly parallel with the order of frequency reported by Pearce and Brown (40) as characteristic of the organs to which their tumor metastasized.

This seems to afford fairly conclusive evidence that the anatomical distribution of metastases, of this tumor at least, is chiefly dependent upon the frequency distribution of embolic tumor cells.

It has been noted in examinations of autopsy specimens that embolic tumor cells lodging in arterioles frequently do not survive, in contrast to those that lodge in capillaries and thin-walled veins (53, p. 173). This observation is also confirmed by the experiment described above, wherein injected cell suspensions produced tumors in those instances only where the cells lodged in capillaries and sinusoids. The spleen, for example, received relatively few embolic cells, and those it did receive lodged chiefly in the tiny, thick-walled arterioles; tumors did not result. This was in contrast to the kidney, where large numbers of embolic cells were arrested, chiefly in the glomerular loops in which location numerous tumors resulted.

Correlated with these observations is the finding that single tumor cells, or clusters tiny enough to penetrate the capillaries, were more successful in producing tumors than were large clumps, because the latter were arrested in arterioles. This is contrary to some reports based upon autopsy material (53, pp. 175, 179).

That the distribution of metastases may vary for different tumors, even though the site of origin of their embolic cells is the same, has been indicated in our own studies (14, 15)[1] as well as by Sugarbaker (46).[2] These observations emphasize only that all tumors are not identical in their behavior. Until we find ways to test critically their subtle peculiarities, we can only speculate as to the reasons for their vagaries.

PROBLEMS IN METASTASIS PECULIAR TO THE LUNGS

There are two points of interest peculiar to the lungs:

1. *The problem of transpulmonary passage of embolic tumor cells.*—It has been suggested (52) that, except for tiny tumor cells, such as lymphoblasts, tumor cells are for the most part arrested in the lung capillaries. If so, secondary growths in other organs that receive emboli through the arteries would depend upon the release of cells from metastic tumors that have become established in the lungs. However, Prinzmetal *et al.* have recently shown (41) that glass beads having a diameter much greater than that of ordinary capillaries are capable of passing readily through the pulmonary circulation. This observation suggests the existence of arteriovenous shunts in the lungs sufficiently large to allow almost any tumor cell to pass through. In confirmation of this, it has been shown that tumor cells considerably larger than lymphoblasts do indeed immediately pass through the lungs. Zeidman (56) found that suspended tumor cells injected into the systemic veins of one animal could be recovered immediately from the aortic blood, and, when injected into a second animal, produced tumors. Three different tumors in two species (rabbits and rats) were used in this study, and in each the cells passed immediately through the lungs. It seems probable, then, that the transpulmonary passage of embolic tumor cells is a more common occurrence than previously supposed.

2. *The lung as a site of growth.*—That the lung may differ from some other organs in its ability to support the growth of metastatic tumors has been demonstrated recently by Lucké *et al.* Tumor cell suspensions introduced into the hepatic and pulmonary circulation produced larger tumors in the liver than in the lungs, and, although more tumors appeared in the lungs, their total mass was less than that of the hepatic tumors (34).

Also, in studies on autopsy material (53, pp. 175, 179), necrotic and degenerating tumor emboli are far more commonly seen in the lung than in the liver.

If any organ does differ from the others in ability to support the growth of secondary tumors, it might, on theoretical grounds, be predicted that the lung would be that organ. The lung is essentially an air-filled sponge, in contrast to the solid structure of other organs; it is continually expanding and contracting, and it is continually subjected to physiological changes in pressure. These factors must in turn affect the circulation of the organ. It is reasonable to suppose that such physical, mechanical, and circulatory events would have some effect upon the establishment of secondary tumors.

Thus, from the evidence at hand, one would conclude that conditions for tumor growth are relatively unfavorable in the lung.

It would seem, at first, paradoxical that the most frequent site of all metastatic tumors, the lungs, should provide the least favorable conditions for the event to occur. This paradox is dissipated when the fact is taken into account that the lung receives the entire systemic venous drainage

[2] Sugarbaker also infers that "organ selectivity" is a major influence in determining the sites of metastases but gives no support for this inference other than the long-established fact that some organs are more often the sites of metastases than are other organs.

and, hence, must be the site of arrest of many more emboli than reach other organs. If only a few of these emboli survived, it would be expected that the lung would still be the organ most commonly the site of metastases.

SUMMARY

Combining the results of the investigations reviewed in this paper, the following concepts relative to the dissemination of malignant tumors within the body are tentatively advanced:

The local invasiveness of cancer cells is primarily dependent upon loss of the mutual adhesiveness of the cells, which loss is associated with, if not due to, local calcium deficiency. The easily detached single cancer cells, or even small clusters of them, are actively ameboid and are thereby enabled to penetrate the adjacent normal tissues.

Distant dissemination of cancer cells, as a sequel to local invasiveness, is largely dependent upon entrance of cells into vascular channels through which they are carried to other parts of the body. Many embolic cells, however, are unable to establish secondary growths in their new location.

Duration of growth of the primary tumor bears a positive relationship to the number of metastases, whereas the size of the primary tumor is less effective in influencing the number of metastases than are other factors as yet undetermined.

The venous distribution of metastases is largely dependent upon the anatomical arrangement of the venous channels (including the vertebral veins) into which emboli are commonly released. This accounts for the frequency of metastatic involvement of the lungs, the liver, and the bones of the spine, pelvis, and skull.

The arterial distribution of metastases is also dependent upon the number of embolic cells reaching the various organs, and in addition depends upon whether the embolic cancer cells lodge in capillaries and sinusoids or in thick-walled arterioles. These phenomena are variable from organ to organ, and evidently account in large measure for the relative infrequency of metastasis to some organs (spleen, muscle) and the frequency of metastasis to other organs (adrenals, kidney).

The lungs, however, although frequently the site of metastatic tumors, apparently afford relatively adverse conditions for tumor growth. This may be attributable to the peculiar physical structure, functions, and circulatory mechanisms of the lungs. The immediate transpulmonary passage of embolic tumor cells is perhaps more common than has been thought in the past.

It is further indicated that single embolic cells or tiny clusters, rather than large clumps, give rise to most metastases because these cells are able to reach the capillary bed where conditions are most favorable for the development of secondary tumors. Larger clumps lodge in arterioles, a situation unfavorable for the establishment of metastatic growths.

In the attempts herein reviewed to reveal "soil" factors as affecting the distribution of metastases, it would appear that they consist chiefly of physical or mechanical circulatory differences rather than of chemically favorable or unfavorable environments for the growth of tumor cells. In view of the results obtained in the experiments here reviewed, it is suggested that before hypothetical "chemical soil" factors are offered in explanation of the distribution of metastatic tumors, simple "mechanical-circulatory" factors affecting the frequency distribution of embolic tumor cells must be eliminated for each tumor and host studied.

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The Effect of Hypophysectomy on the Development of Adrenal Tumors in C3H Mice*

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Adrenal cortical adenomas occur after gonadectomy in several inbred strains of mice (13, 14). Pituitary control of the adrenal cortex suggests that adenomatous changes in the cortex may also depend on pituitary hormonal influence. The present experiment deals with the effect of hypophysectomy on the development of postcastration adrenal changes in C3H mice. Since it was necessary to have pair-fed controls, some effects of partial inanition on the adrenals and other organs, as compared to the effects of hypophysectomy, were also observed.

Casas *et al.* (1) found that 33 and 50 per cent caloric restriction decreased but did not prevent adrenal cortical hyperplasia in ovariectomized C3H mice. Woolley (12) reported that castrated DBA mice did not respond well to hypophysectomy, but over a short period of time they did not develop nodular hyperplasia of the adrenals.

METHODS

C3H strain mice were used; they were 3 months old at operation. Castration and hyophysectomy were performed in 23 females and ten males, which were given Purina Fox Chow and water ad libitum. Castration and sham hypophysectomy, including removal of the bone plate, were carried out in equal numbers of animals, which were fed such daily weighed portions of fox chow that each animal matched one of the hypophysectomized animals in body weight. The average daily intake of hypophysectomized mice was 1.6 gm. The controls, which were much more active, required 1.9 gm. to maintain the same weight. Intact mice of the same age, given free access to food, consumed 4 gm/day.

Hypophysectomized animals and their controls were sacrificed at fairly evenly spaced intervals from 56 to 400 days after operation. Evidence of completeness of hypophysectomy was observed by the continuing loss of body weight in all animals during the experiment, absence of pituitary fragments at post mortem (using a dissection microscope), and absence of recognizable pituitary tissue in serial sections of the decalcified sellar region in five representative animals.

Body weights were obtained weekly, and vaginal smears on 3 consecutive days per month. At post mortem the principal organs were weighed. Serial sections at 5 μ were made of all adrenals.

Comparable observations were made on three to six mice of each sex in the following groups: (*a*) intact, 3–12 months of age; (*b*) hypophysectomized but not castrated; (*c*) castrated, sham-hypophysectomized, and fed ad libitum.

RESULTS

The most striking observation was the complete absence of large, pale-staining cells ("type B," Woolley and Little [14]) in the adrenal cortices of castrated-hypophysectomized mice and the presence of nests of these cells forming nodular hyperplasia, or adenomas, in all but one of the controls (Table 1). The one control mouse without hyperplasia was sacrificed 57 days after operation. Groups of small, darkly staining ("type A") cells, lying just under the capsule and sometimes penetrating into the middle of the cortex, were always present in castrated-hypophysectomized female mice, and in most males. According to Woolley and Chute (13), the criterion for the prevention of adrenal changes is the nonappearance of type B cells. The histological changes in the adrenals of the control mice were similar to those described by Casas *et al.* (1) in castrated C3H mice which were restricted to 67 per cent or less of their normal caloric intake. In the present instance, the caloric restriction was a little over 50 per cent. The added operation of sham-hypophysectomy had no detectable permanent effect on the mice, either in regard to general health or the state of the adrenals. When such animals were given unlimited food they grew to normal size and weight.

In the control mice, after 2 months from the time of operation, there were small whitish or yellow opacities grossly visible just under the capsules of both adrenals. Microscopically, globular collections of the lipid-containing type B cells arose first in the outer part of the cortex in association with type A cells. Later the whole thickness of the cortex was involved, usually in two or three separate places in each cross section, and nodules of the abnormal cells bulged on the surface of the gland and into the medulla (Figs. 1, 2, 4, and 7).

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In the castrated, sham-hypophysectomized controls without dietary restriction, the adenomatous process in the adrenal was similar in kind but more extensive than in the animals under food restriction (Figs. 1 and 7, compared to Figs. 2 and 4). No carcinomas were found.

Groups of type A cells were seen in the outer cortex of normal female C3H mice at 6 months of age. These cells also occurred in hypophysectomized, noncastrated mice, in which the adrenal cortex was apparently indistinguishable histologically from that found in hypophysectomized-castrated animals. In both these groups, a concentration of smaller, darkly stained cells in the innermost zone of the cortex was conspicuous, while it was absent or much less prominent in the controls, as may be seen in the figures. This area is usually referred to as the X zone.

In the hyperplastic areas of the adrenal cortex shown in Figures 2 and 4, there were areas of "brown degeneration" near the medulla, where masses of pigmented material filled the cells and coalesced. Frantz and Kirschbaum (4) suggested that this process, which consists of deposition of a brown ceroid pigment, was a degenerative alteration associated with aging. They observed it particularly in steroid-secreting cells of the adrenal cortex, ovary, and testes.

There is no evidence that the hypophysectomized mice or the controls under food restriction in the present experiment produced a significant amount of estrogenic hormone. Vaginal smears and sections were invariably castrate in type. The controls which were not under food restriction showed continuous sub-estrous smears after 2-3 months from the time of operation, presumably due to estrogen secreted by the adrenal adenomas.

The adrenal cortex showed very little atrophy in the underfed controls, compared to that occurring after hypophysectomy (see figures and Table 2), while the medulla was little changed in either group. The organ weights given in Table 2 indicate that there was a much more profound atrophy of the heart, liver, and kidneys in the hypophysectomized animals than in the controls fed to the same body weight. The thymus, on the other hand, and possibly the spleen, were heavier in the hypophysectomized mice. Histologically, the cells of the organs weighing less in the hypophysectomized mice appeared to be smaller, and contained less cytoplasm than in the controls. These data indicate significant activity of the pituitary during a state

TABLE 1

BODY WEIGHT AND INCIDENCE OF NODULAR HYPERPLASIA OF THE ADRENAL CORTEX

Mice of the C3H strain were used. The castrated, sham-hypophysectomized controls were pair-fed to the same body weight as that of the castrated, hypophysectomized mice

	Hypophysectomy						Sham-hypophysectomy					
	No. mice	Av. wt. (gm.) Op.	Av. wt. (gm.) Fin.	Days postop.	Cortex A*	Cortex B†	No. mice	Av. wt. (gm.) Op.	Av. wt. (gm.) Fin.	Days postop.	Cortex A*	Cortex B†
Female	23	19.4	13.5	77–400	23	0	23	18.0	13.6	79–396	23	23
Male	10	22.1	14.2	56–248	6	0	10	22.2	14.5	57–249	10	9

* Number of mice in which groups of small, darkly stained, type A cells were found in the adrenal cortex.

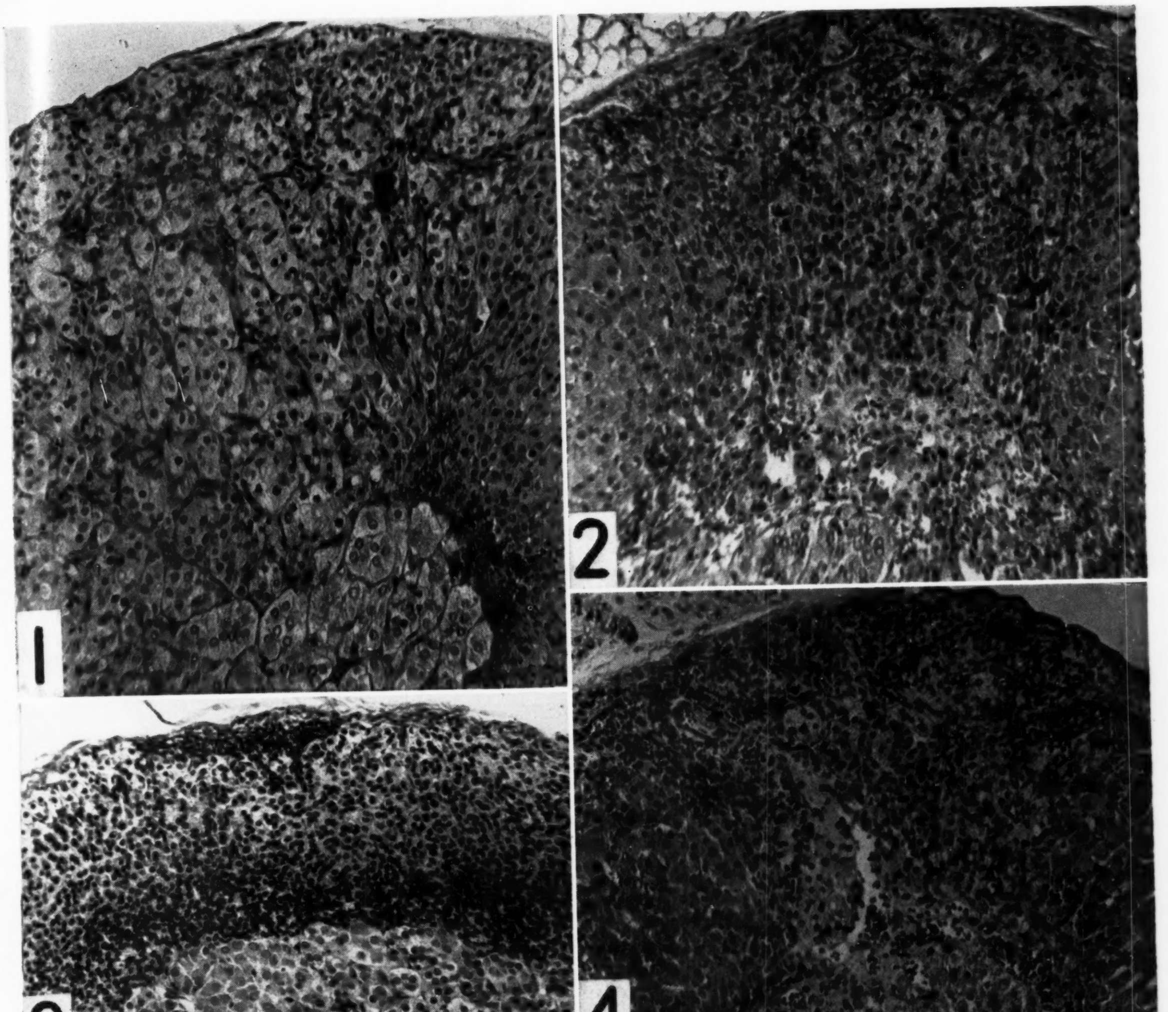
† Corresponding figure for nests of large, pale-staining, type B cells. These constitute the nodular hyperplasia or adenomas.

TABLE 2

ORGAN AND BODY WEIGHTS

The data from nine castrated-hypophysectomized female mice, autopsied 150–400 days after operation, are compared to corresponding data from nine sham-hypophysectomized, castrated control animals, which were underfed to the same body weight

Organ	Hypophysectomy Mean wt.	Hypophysectomy S.D.	Sham-hypophysectomy Mean wt.	Sham-hypophysectomy S.D.	$p<$
Adrenal (mg.)	0.73	0.15	1.97	0.23	0.01
Kidney (mg.)	62	3.5	109	8.6	0.01
Liver (mg.)	463	51	734	151	0.01
Heart (mg.)	38	3.9	66	2.7	0.01
Thymus (mg.)	10.7	2.1	2.3	0.9	0.01
Uterus (mg.)	2.7	0.86	3.0	0.95	
Spleen (mg.)	27	4.0	21	6.2	
Body wt. (gm.):					
At operation	19.1		18.2		
At autopsy	13.1		13.5		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Photomicrographs of mid-sections of mouse adrenals. Hematoxylin and eosin stain. Mag. $\times 150$.

FIG. 1.—Female, castrated, sham-hypophysectomized, full-fed, 164 days after operation. Nests of pale-staining type B cells extend across the entire cortex and protrude into the medulla. Normal cortex to the right. Nuclei of nodule are darker than those of medulla.

FIG. 2.—Female, castrated, sham-hypophysectomized, underfed, 214 days after operation. Groups of type B cells, mingled with type A cells, extend through the cortex. Near the medulla are areas of brown degeneration. Normal cortex on both sides.

FIG. 3.—Female, castrated-hypophysectomized, 157 days after operation. Atrophy of cortex. Type A cells present.

FIG. 4.—Male, castrated, sham-hypophysectomized, underfed, 213 days after operation; control for mouse in Figure 6. Groups of both A and B cells extend across the cortex. Normal cortex on both sides.

FIG. 5.—Female, castrated-hypophysectomized, 210 days after operation. Similar to Figure 3, but with more atrophy. The medulla remains unchanged. Underfed control shown in Figure 2.

FIG. 6.—Male, castrated-hypophysectomized, 230 days after operation. Atrophy of cortex with no type A or B cells.

FIG. 7.—Male, castrated, sham-hypophysectomized, full-fed, 146 days after operation. Nests of type B cells extend across the cortex. Only part of the extent of the nodule is cut in this section. Adjacent normal cells are compressed.

of partial starvation in which body weight was maintained at less than one-half of normal for mice of the same age and strain in this laboratory.

DISCUSSION

Spontaneous adrenal cortical tumors are rare in human beings and in most if not all other species. Hyperplastic adrenal changes appear after gonadectomy in mice, guinea pigs (11), and hamsters (5). The spontaneous tumors in humans, as well as those induced in mice and guinea pigs, are capable of secreting androgen or estrogen. The administration of adrenocorticotropic hormone may cause the adrenal to secrete sex hormones in the absence of the gonads (7, 10). Apparently, the adrenal cortex, having a close embryological association with the gonads, retains a latent capacity to assume some of the gonadal function when stimulated by the pituitary. Removal of the gonads may act to promote adrenal hyperplasia by removing an inhibitory effect of sex hormone on the pituitary, or by releasing to the adrenals the pituitary hormone normally absorbed by the gonads.

The absence of adenomatous changes in the adrenals of castrated and hypophysectomized mice and their occurrence in controls fed to the same body weight are evidence that pituitary secretion is necessary for development of these tumors.

It has been found that large doses of estrogen prevent pathologic changes in the adrenals of mice after castration (6, 15), but "physiological" doses of estrogen, estrogen plus progesterone, or testosterone (3) do not prevent adenoma formation, at least in gonadectomized NH strain mice. The production of adrenal cortical tumors in rats by injection of growth hormone (8), and in one mouse by injection of adrenocorticotropic hormone (2), is perhaps significant as to which pituitary hormones are involved, but the experiments were done in nonhypophysectomized animals.

A state of "pseudo-hypophysectomy" resulting from inanition has been described (9), and evidence of depressed pituitary function has often been observed during starvation. Statistically significant differences in the weights of heart, kidney, liver, adrenal, and thymus between hypophysectomized mice and control mice pair-fed to maintain equal weight prove that the pituitary gland exerts important physiological actions even in extreme caloric deficiency.

SUMMARY

1. The development of postcastration adrenal cortical nodular hyperplasia in male and female C3H mice is prevented by hypophysectomy.

2. Food restriction in castrated and sham-hypophysectomized mice, fed so as to maintain the same body weight as that of castrated-hypophysectomized animals, does not prevent nodular hyperplasia in the adrenal cortex, though it does prevent evidence of estrogen secretion from the adrenal.

3. Significant differences in organ weights, between hypophysectomized mice and underfed controls with the same body weight, indicate that some important pituitary functions persist in spite of severe dietary restriction.

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Studies on the Origin of the Naturally Occurring Antibodies against Tumor Viruses Developing in Aging Chickens*

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It has been established (10) that, concomitantly with growth and aging, there develops in the blood of many apparently normal chickens neutralizing antibodies for viruses of avian tumors. In earlier studies (10) and in a subsequent investigation by Freire and Duran-Reynals,[1] antibodies against the Rous virus were found in 72 of a total of 87 adult birds tested. Similar antibodies were also detected in adult fowls against the viruses of the Fuginami sarcoma and the Mill Hill 2 endothelioma (10). On the other hand, there were no antibodies against the Rous virus in the blood of 67 chicks from 1 to 52 days of age (10).[1]

These facts, which, in avian cancer, parallel what is observed in so many infectious diseases, may well be considered an indication of a subclinical infection of the chickens, either by the tumor viruses themselves or by other viruses antigenically related to them. If this is true, the viruses are to be considered exogenous agents; and, consequently, if it were possible to eliminate them from the environment in which the birds live, no antibodies would be detected in the birds throughout their lives. However, some workers still prefer to believe that the tumor viruses are of endogenous origin. Thus, it would seem logical to assume that, whatever opinion these workers may have on the nature of the virus neutralizing factors, the presence of these factors should be little affected, if at all, by the environment in which the birds grow to mature age.

The chickens used in the study of lymphomatosis carried out during the last 12 years in the U.S. Regional Poultry Research Laboratory at East Lansing, Michigan, provided a unique opportunity to decide which of the two hypotheses is right. At the East Lansing Laboratory the main flock of White Leghorn chickens is comprised of several inbred lines. Although these chickens were raised under ordinary conditions, they were free from all the usual poultry pathogens except coccidiosis and lymphomatosis. Therefore, these "contaminated" birds were exposed to the viruses of the latter disease and those of other naturally occurring tumors from the time they were hatched until they were destroyed or died. On the other hand, chickens of one of these inbred lines (line 15) were hatched and reared as family units (e.g., the offspring of a specific dam and sire) in isolated pens. Thus, these "isolated" chickens were directly exposed to only their own brothers and sisters, and were indirectly exposed to whatever may have accidentally been introduced via the caretaker, feed, and air (7, 23, 24). Lymphomatosis occurred in some of these isolated pens, but the incidence was significantly lower than that observed in the main contaminated flock. The main source of infection from lymphomatosis in the isolated birds was believed to have been via egg transmission, as Waters has pointed out (23–25). This view is supported by the recent work of Cottral *et al.* (7–9), which indicated that certain normal-appearing hens are nevertheless carriers of the lymphomatosis virus and can transmit the virus to their offspring.

Sarcomas of the Rous type were not found in the isolated birds at the time this study was carried out. However, such tumors have since occurred in similarly isolated chickens. In the contaminated population, sarcomas of the Rous type have been more frequently observed, but in comparison with lymphomatosis, the incidence of sarcomas is very low (7).

Sera from both contaminated and isolated chickens were tested for the presence of neutralizing antibodies for the Rous sarcoma virus. This paper reports the results from these tests, as well as from complementary experiments on the same animal material.

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† Bureau of Animal Industry, Agricultural Research Administration, U.S. Department of Agriculture.

[1] P. M. Freire and F. Duran-Reynals, unpublished observations.

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MATERIALS AND METHODS

The sera were secured from the years 1948 to 1951. They were sent, frozen, from Michigan to New Haven, were slowly thawed, and kept in the refrigerator. They were generally tested from 1 to 2 months after being collected. In some instances, the sera had been kept frozen for much longer periods of time, but there is no reason to suspect that this procedure had any effect on their virus-neutralizing properties. The age of the chickens, except in two cases, ranged roughly from 300 to 900 days.

The main feature of the method for the detection of neutralizing antibodies was the incubation of serum dilutions and sarcoma extracts at low temperature, a procedure which allows neutralization with minimal deterioration of the virus (10). This method was further improved by Freire and Duran-Reynals in a study on immunity to the Rous sarcoma virus.

The sarcoma extracts were secured by extracting the pooled tissue from three tumors about 12 days old, induced by inoculating 2–3-week-old Plymouth Rock chicks, intramuscularly, with cell suspension of the sarcoma. These tumor extracts were prepared at the concentration of 1:20 in saline, and were centrifuged at 2,000 r.p.m. for 20 minutes. The supernatant fluid was further diluted by adding 10 more volumes of saline.

The sera were diluted so that, when mixed with equal volumes of the sarcoma extract at 1:200, the following concentrations of serum resulted: 1:2, 1:6, 1:20, 1:63, 1:200, and 1:632. The sera and the extracts remained in contact at 4° C. for from $3\frac{1}{2}$ to 5 hours before they were inoculated.

Ten Plymouth Rock chicks about 2 weeks old were used for titrating each serum. Each chick was inoculated intradermally into the six following sites: the ventral surface of the forewings, both sides of the breast, and the outer surface of the thighs. Five of the inocula were mixtures of virus and different serum dilutions, while one inoculum was a control of the virus preparation alone, at 1:400. The latter substituted for one of the serum mixtures, a different serum dilution being skipped in each chick. The amount of inoculum was 0.2 cc. Accidental losses often reduced considerably the number of chicks available for final analysis.

Six sera were generally titrated at the same time against the same tumor extract, care being taken to titrate simultaneously sera from isolated and from contaminated chickens, or sera from chickens before and after immunization against a lymphomatosis antigen.

The areas of the resulting tumors were recorded 30 days after inoculation or, before this time, at death of the chicks. Chickens that died before 12 days were not taken into consideration. There was no difficulty in evaluating the cases where total neutralization of the virus was obtained, since no tumor growth was detected in the sites injected with the serum-virus mixtures, while the control areas were positive. In the cases where some growth occurred in the serum-virus mixtures we adopted as a threshold value half the mean area of the control tumors of all the chicks injected with a given tumor extract. Thus, if the tumors induced by the serum-virus mixture were equal to or larger than this mean area, the results were considered as a positive response, indicating that the serum was not neutralizing; and, if the tumors were smaller than this area the sera were considered as neutralizing.

In estimating the titers of partial neutralization for each serum we have taken into consideration only those cases where the number of positive responses was half or less than half the total number of tumors. Thus, results such as $\frac{3}{4}$ or $\frac{2}{3}$ were not considered as indicating neutralization, but results such as $\frac{1}{2}$, $\frac{2}{4}$, and $\frac{1}{3}$ were considered as indicating neutralization. We resorted to this rather arbitrary procedure as a result of the lack of uniformity in the variation of the areas of the tumors at different levels of serum concentration. However, even following this procedure, minor discrepancies in some of the readings occurred, as is seen in Table 1.

In this table are the direct readings in some representative cases. The first three sera come from contaminated flocks; serum H 206 W is a case of total neutralization at the titer of 1:632, serum H 268 C_2 is a case of total neutralization at 1:6 and partial neutralization at 1:63; serum H 621 K_2 is also considered as a case of total neutralization despite the small number of surviving chicks. In the tests on chicken H 1313 I (Table 2), and chickens H 476 A, H 482 A, and H 621 K2 (Table 3), there also was a small number of surviving chicks. Serum D 821 A_2, from a chicken raised in isolation, and serum H 306 V, from a chicken affected with neurolymphomatosis, are cases showing complete lack of neutralization. Serum F 355 U_2 is also considered as totally nonneutralizing, despite the results obtained in the last two serum dilutions.

The procedure used for the antibody titration in the Laboratories at East Lansing was similar to those used at New Haven but with the following differences: A large pool of frozen virus preparation was stored at −76° C., its potency titrated, and this was used for all neutralization tests at a dilution of 1:100. Serum samples were heated to 50° C. for 30 minutes, and dilutions of 1:5, 1:25, 1:125, and 1:625 were used. Each bird was inoculated at eight different sites—namely, right and left forewing, wing, breast, and leg. Kenzy (20, 21) in his studies on chicken sera from field flocks, followed a comparable method.

TABLE 1

REPRESENTATIVE RESULTS IN THE TITRATION OF SERA

SERUM DILUTIONS	SERA TESTED*		
	H 206 W	H 268 C_2	H 621 K_2
1:2	0/4	0/5	0/2
1:6.3	0/3	0/5	0/2
1:20	0/4	1/5	0/1
1:63.2	0/3	3/6	0/2
1:200	0/3	3/4	0/2
1:632	0/3	2/5	0/1
	D 821 A2	H 306 V	F 355 U_2
1:2	3/4	6/7	2/2
1:6.3	3/3	5/5	2/3
1:20	3/4	6/6	2/3
1:63.2	3/4	6/6	2/3
1:200	2/2	5/5	1/2
1:632	2/3	6/6	1/2

* Number tumor takes/ total number of sites used.

Other experiments of the present investigation involved immunization of chickens with lymphomatosis material for the purpose of studying whether such treatment induced the production of antibodies against the Rous virus or elicited a resistance to the inoculation of the virus into the treated birds. In these experiments, largely carried out in East Lansing, the transplantable lymphocytoma RPL 12 was employed. The history of this tumor and the methods followed for the preparation of the immunizing inocula, either cell-free or containing cells, have been given elsewhere (5, 6). For testing the chicks against the Rous sarcoma, the tumor was minced, diluted with buffered saline, mixed in a Waring Blendor, rendered cell-free by centrifugation, and then frozen and stored until used. Each chick received the virus intradermally as follows: 0.2 cc. of a 1:10 dilution into the left wing, breast, and thigh; and 0.2 cc. of a 1:100 dilution into the right wing, breast and thigh.

For testing against the lymphoid tumor a cell suspension was prepared by mincing breast tumors from donor birds previously inoculated with tumor RPL 12. The cells were di-

luted with sterile saline and filtered through sterile gauze. Each bird received 0.2 cc. inoculated intramuscularly in the right side of the breast.

EXPERIMENTAL

Neutralization of the Rous sarcoma virus by sera from chickens raised in isolation and chickens raised in contaminated surroundings.—Results of the titrations of 26 sera from chickens of flocks hatched and reared in isolated surroundings are given in Table 2, and results of titrations of 24 sera, from chickens reared in contaminated surroundings, are given in Table 3. All the isolated chickens, and the last nine contaminated chickens (from number 13 to 24), belonged to inbred line 15. The rest of the contaminated chickens belonged to other inbred lines. The incidence of lymphomatosis in isolated and contaminated flocks is also given in the tables.

The following is clearly seen from the tables: (*a*) only four of the sera from isolated chickens showed partial neutralization of the Rous sarcoma virus and then at a low titer; and (*b*) only three of the sera from contaminated chickens failed to neutralize the virus, the neutralization being total in three cases and total or partial in another five cases at all serum dilutions.

Neutralization of the Rous sarcoma virus by sera from chickens reared in isolation after experimental immunization with lymphomatosis antigen.—In view of the above results it was logical to suspect an antigenic relation between lymphomatosis and sarcoma viruses. To test the hypothesis, experiments were devised to determine whether experimental immunization against lymphomatosis brought about the development of antibodies against the Rous sarcoma virus.

Accordingly, five adult chickens reared in isolation were given several intravenous injections of a cell-free extract of a transmissible lymphoid tumor (RPL 12). These tumors are indistinguishable from tumors regularly obtained in cases of visceral lymphomatosis (4, 6). Sera were collected before and after the first and second series of injections. These are the first five chickens listed in Table 4.

TABLE 2

INCIDENCE OF NEUTRALIZING ANTIBODIES AGAINST THE VIRUS OF ROUS SARCOMA IN NORMAL CHICKENS FROM FLOCKS REARED IN ISOLATION

Source of sera	Incidence of lymphomatosis to 600 days of age: Penmate sibs (isolated flock) (per cent)	Controlled sibs (contaminated flock) (per cent)	Titer of neutralizing antibody: Total neutralization	Partial neutralization
D 805 Q2*	0.0	44.2	0†	1:2
D 821 A2	"	"	0	0
D 821 K2	"	"	0	1:2
H 1313 C	10:0	Not determined	0	0
H 1313 E2	"	" "	0	0
H 1313 I	"	" "	0	0
H 1310 X	"	" "	0	0
H 1310 W	"	" "	0	0
I 1401 R3	17.6	74.4	0	0
I 1410 A4	"	"	0	1:20
I 1412 G3	"	"	0	0
I 1412 J3	"	"	0	0
I 1412 M3	"	"	0	0
K 1473 O3	Not determined	Not determined	0	1:25‡
Pool 1§	" "	" "	0	0
Pool 2	" "	" "	0	0
Pool 3	" "	" "	0	0
Pool 4	" "	" "	0	0

* Wing band number of chicken.
† "0" means no dilutions showing neutralization.
‡ Titration carried out at East Lansing
§ Each pool contains the sera from three different chickens.

Twelve other chickens arising from the same source were divided into four lots of three and were injected with four different preparations of the same lymphoid tumor strain. To each of the preparations formalin was added to make concentrations of 0.05 and 0.2 per cent. After twelve injections the birds were bled, and sera from the three birds of each group were pooled. Neutralization titration for the four groups of pooled sera are presented in Table 4. Results are also given for sera of three chickens of genetic lines other than line 15 which were raised with the main flock of chickens. These received repeated injections of active tumor extract. Results of the titrations presented in Table 4 show that immunization of isolated stock with the nonformalinized antigen resulted in every case in a development of antibodies

which neutralize the Rous sarcoma virus, whereas chickens of the same stock which received formalinized antigen failed entirely to do so. It is of interest that the sera of chicken H 1310 W and of H 1310 X, which were tested after two injections and again after a booster injection, showed a distinct rise in antibody titer between the second and third injection. Results further indicate that chickens of the main flock which had a high level of exposure to lymphomatosis had Rous virus antibodies before hyperimmunizing injections were given and apparently did not respond to further exposure to the active agent.

Experiments on cross-immunity between a transplantable lymphoid tumor and the Rous sarcoma.—As a natural sequence to the above tests, we tried

TABLE 3

INCIDENCE OF NEUTRALIZING ANTIBODIES AGAINST THE VIRUS OF THE ROUS SARCOMA IN NORMAL CHICKENS FROM CONTAMINATED FLOCKS

Source of sera	Incidence of lymphomatosis in respective sibs to 600 days (per cent)	Titer of neutralizing antibody: Total neutralization	Partial neutralization
H 206 W	39.0	1:632	
H 268 C2	"	1:6	1:63
H 270 A2	"	0	1:6
H 476 A	"	1:63	1:632
H 482 A	"	0	1:632
H 621 K2	"	1:632	
H 475 A	"	1:6	1:20
H 310 M2	"	1:632	
H 721 N	"	1:20	1:200
I 530_2	15.6*	1:63	
I 678 O	"*	0	0
I 679 V	"*	1:2	
J 1301 Y2	32.8	1:6	1:63
J 1301 X2	"	1:2	1:632
J 1313 N2	"	1:2	1:632
J 1317 O	"	1:20	1:63
J 1401 H2	"	1:2	1:63
J 1414 B	"	1:6	1:200
J 1301 E3	"	0	0
J 1301 F3	"	0	1:63
J 1314 N2	"	1:2	1:20
J 1316 D4	"	0	0
J 1317 S	"	1:20	1:63
J 1407 W	"	1:20	1:632

* To 300 days.

to ascertain whether immunization of chickens against lymphomatosis resulted in an active immunity against the Rous sarcoma.

Accordingly, one group of 51 White Leghorn chicks from isolated inbred line 15 were inoculated intraperitoneally at 1 day of age with 0.2 cc. of a cell-free virus material prepared from tumor RPL 12 (5), while another group of 48 chicks of the same source and age were inoculated intraperitoneally with 0.2 cc. of heparinized whole blood from a hen (J404A) which was suspected of being a carrier of lymphomatosis virus. Thirty days later, the above birds, and also 58 control chicks of the same age, were given a challenging inoculation of the Rous virus.

The results obtained are summarized in Table 5. It is seen that the previous inoculation of the lymphoid tumor material delayed and partially prevented the development of the Rous tumor, whereas, the inoculation of the blood of the hen suspected of being a carrier was ineffective.

In another experiment two groups of chicks were inoculated with lymphoid tumor cells (RPL 12). Most of these chickens developed lymphoid tumors which subsequently regressed. The chicks that survived were then given a challenging dose of the same tumor strain. Most of the chicks were found to be immune. The same chicks were then given an inoculation of the Rous virus, with the result that most of them developed sarcomas. Details and results of the experiment are given in Table 6. It is clear from the results that, while treatment almost entirely protected the birds against inoculation of the malignant lymphoid cells, it did not afford any protection against the sarcoma virus. The methods followed in the tests have been described before in the text.

TABLE 4

DEVELOPMENT OF NEUTRALIZING ANTIBODIES IN CHICKENS AGAINST THE VIRUS OF THE ROUS SARCOMA FOLLOWING IMMUNIZATION INJECTIONS WITH LYMPHOMATOSIS MATERIALS

Source of sera	Titer of neutralizing antibody: Before immunization, Total	Before immunization, Partial	After immunization, Total	After immunization, Partial
I Isolated chickens immunized with active antigen:				
H 1310 W	0	0	1:6†	1:10
"			1:20‡	1:200
" *	0	0	1:5‡	1:125
H 1310 X	0	0	1:6†	1:10
"			1:20‡	1:200
" *	0	0	1:5‡	1:125
I 1401 R3	0	0	0	1:10
I 1412 J3	0	0	1:10	1:100
K 147303*	0	1:25	1:25	1:125
II Isolated flock chickens immunized with formalinized antigen:				
Pool 1	0	0	0	0
2	0	0	0	0
3	0	0	0	0
4	0	0	0	0
III Main flock chickens immunized with active antigen:				
J 258 K*	1:5	1:125	1:25	1:125
K 669 G2*	1:25	1:25	0	0
K 669 L2*	0	0	1:25	0

* Titrations carried out at East Lansing.
† Sera collected 11/29/48 after injection on 11/2/48 and 11/9/48.
‡ Sera collected 4/5/49 after injections on 3/23/49

Neutralization of the Rous sarcoma virus by sera from chickens affected with naturally occurring lymphomatosis.—The tests were carried out on sera from six chickens affected with either neural or

visceral lymphomatosis. The results in Table 7 show that sera of two out of three birds with visceral lymphomatosis did neutralize the virus, whereas sera from another three birds affected with neurolymphomatosis were entirely ineffective. One must point out, however, that chickens 1 and 3, affected with neurolymphomatosis, were only 67 and 94 days old, respectively; and this fact may, at least partly, explain the lack of neutralizing power of their sera.

TABLE 5

THE PARTIAL SUPPRESSION OF THE ROUS TUMOR VIRUS ACTIVITY BY THE PRIOR INOCULATION WITH CELL-FREE PREPARATIONS OF A LYMPHOID TUMOR

Source of lymphomatosis virus inoculated	No. chickens	Percentage of chickens that developed sarcomas at intervals after inoculation with Rous virus: 12 days	21 days	29 days	37 days
RPL 12 (L 10)	51	5.9	54.0	66.7	71.6
Blood of J 404 A	48	92.1	93.8	93.8	93.8
None	58	89.7	94.8	94.8	94.8

Lymphomatosis agent administered when chicks were 1-day-old; Rous virus was given 30 days later.

TABLE 6

TEST OF CROSS IMMUNITY BETWEEN TRANSPLANTS OF LYMPHOID TUMOR STRAIN RPL 12 AND THE VIRUS OF ROUS SARCOMA

Group	Initial treatment: Inoculated with lymphoid tumor cells: No. birds	Age at inoc. (days)	Per cent lymphoid tumors	Successive treatments of survivors: Challenged with lymphoid tumor cells: No. birds	Age at inoc. (days)	Per cent lymphoid tumors	Challenged with Rous virus: No. birds	Age at inoc. (days)	Per cent Rous sarcoma
No. 1	30	38	100.0	30	108	0.0	30	114	80.0
No. 2	35	117	97.2	28	151	7.1	23	175	91.0
Control	—	—	—	—	—	—	5	175	80.0

TABLE 7

INCIDENCE OF NEUTRALIZING ANTIBODIES AGAINST THE VIRUS OF THE ROUS SARCOMA IN CHICKENS AFFECTED WITH NATURALLY OCCURRING LYMPHOMATOSIS

Source of sera	Type of disease	Titer of neutralizing antibody: Total neutralization	Partial neutralization
H 306 V	Neural lymphomatosis	0	0
G 124 M	" "	0	0
H 310 M	" "	0	0
F 355 U2	Visceral lymphomatosis	0	0
G 125 O	" "	1:6	1:632
G 159 S	" "	0	1:632

DISCUSSION

In orthodox epidemiology, if antibodies against an ordinary virus are present in the majority of apparently normal individuals from an animal population living under usual conditions, and if these antibodies are absent in individuals of the same population living in conditions of isolation, one concludes without hesitation that, in the former case, the animals have been subclinically infected by the virus itself or by other viruses antigenically related—and also that the responsible virus is obviously of exogenous origin, and is highly prevalent.

This is, essentially, the situation found in our study—the host being the chicken and the agent against which antibodies are developed being a cancer virus, that of the Rous sarcoma. The main point to debate, therefore, is whether the ubiquitous virus is that of the Rous sarcoma (and allied tumors) or viruses causing other conditions, but antigenically related to the sarcoma viruses.

As to the first hypothesis, it could be argued that, in view of the near universality of infection, as indicated by the great number of birds showing antibodies, one should also expect a high incidence of sarcomas in the contaminated flocks—an event opposite to what actually takes place—and, further, that no obvious cases of Rous tumor transmission had been observed in normal chickens living in close contact with tumor-bearing birds. However, it is possible that, as conceivably may occur in the case of Pekin ducks,[2] many birds could acquire a latent infection of the Rous virus by contact and never develop clinically obvious sarcomas. Therefore, a latent sarcoma virus infection may be present in the birds in the main, contaminated flock, and the quarantine procedures may have prevented the introduction of this virus into the isolated pens.

As to the second hypothesis, although viruses or other pathogens can be present in the contaminated flock, theoretically sharing the antigenic

[2] In these birds tumors do not develop frequently, yet their blood has a strong neutralizing effect for the duck variants of the Rous sarcoma (13, 21).

properties of the sarcoma viruses, one should suspect, among them, those that by their effects on cells behave in a manner more nearly similar to the sarcoma viruses. Thus, the idea that the lymphomatosis viruses themselves could be the responsible agents naturally suggested itself, since these viruses are known to be both neoplastic and contagious—not only under natural conditions but also in experiments wherein the disease has been artificially induced (3, 7, 23).

Gross tumors typical of lymphomatosis were entirely absent in one pen of isolated birds, and the incidence of these tumors was very low in the other pens of isolated birds. Thus, it could follow that these birds were comparatively free of the lymphomatosis viruses, and for this reason showed no significant neutralizing antibody titers, whereas the birds in the main population, in the contaminated environment, were subjected to a greater level of exposure, and, therefore, the majority had neutralizing antibodies. This hypothesis is further strengthened by the report of Kenzy (19), in which he has shown that there is an apparent direct relationship between the flock incidence of lymphomatosis and the number of birds in the flocks that show antisarcoma antibodies. However, the fact remains that some of the penmates of the tested chickens did have lymphomatosis, and, in view of the highly contagious nature of the disease and the fact that it can be transmitted in a latent form from parents to offspring through the egg (7, 8), it would seem unlikely that one could so consistently select for testing only the virus-free birds from these pens.

To confirm conclusively the second hypothesis, it would have to be proved that lymphomatosis and sarcoma viruses possess antigens in common. Suggestive confirming evidence is the fact that isolated chickens immunized with materials from lymphoid tumors developed antibodies against the Rous virus, while these antibodies failed to appear when the inoculated tumor material was treated with formalin. However, a latent sarcoma virus could have been present in the lymphoid tumor, thus accounting for the development of the antisarcoma antibodies.

The tests devised to show an active resistance against the Rous sarcoma by immunization of chicks against lymphomatosis did not bring convincing evidence in either way. In one experiment in which cell-free lymphoid material was used for immunization the sarcoma growth was definitely retarded—the result suggesting either virus interference or development of specific resistance. Yet, in another experiment, chickens treated with lymphoid tumor cells proved to be solidly immune to reinoculation of cells of the same lymphoid tumor; but they were highly susceptible to the sarcoma virus. The latter result, however, does not prove that lymphomatosis and Rous viruses are unrelated, because it has been shown elsewhere that immunity to implanted lymphoid tumor cells does not confer immunity to the virus of visceral lymphomatosis (4, 6).

Mention should be made of two other points in connection with the hypothesis that lymphomatosis viruses are responsible for the antisarcoma antibodies: The three birds that had neural lymphomatosis did not show neutralizing antibodies; however, Kenzy (19) has found such antibodies in field cases of neural lymphomatosis. The other point is that the findings on the resistance or susceptibility of the various inbred lines of the Regional Laboratory chickens to naturally occurring and artificially induced lymphomatosis did not parallel the results obtained when chickens from these lines were inoculated with Rous virus (26). However, these results do not necessarily detract from the hypothesis. Greenwood and Carr (18), on the basis of a similar observation, have speculated on the possibility of a connection existing between Rous sarcoma and fowl paralysis.

It is clear, therefore, that no final conclusions can be drawn regarding the nature of the contagious agent. If it were shown that lymphomatosis viruses can mutate into sarcoma viruses, the fact would further confirm what is already known about the variability of avian tumor viruses: first, the variety of malignant lesions—including lymphomatosis—obtained by infection of heterologous (11, 12) or homologous species (15, 16, 19, 22) with sarcoma viruses; and, second, the antigenic relations existing between chicken sarcoma viruses among themselves (1), including those supposedly present in chemically provoked sarcomas (2, 14, 17), and with their variants in other species (13).

Whatever the outcome of future work on the problem, the following can be finally stated: the studies carried out in the Regional Laboratory at East Lansing have conclusively shown the contagious nature of lymphomatosis. Since the disease, as accepted by many workers, has some typical features commonly associated with malignancy, the above conclusion precludes principles which seemed immutably established in the history of neoplasia (8, 12). The present study, carried out on the same animals that served to establish this conclusion, has shown that there are contagious agents responsible for the development of antibodies against viruses which are today accepted by everyone as being the cause of genuine cancers. This makes it extremely probable, if not

entirely certain, that these contagious agents, either by themselves or after undergoing variation, are the cause of the cancers.

SUMMARY

Most sera from 26 chickens hatched and reared in isolation and showing an absence or a low incidence of lymphomatosis were devoid of neutralizing antibodies for the Rous sarcoma virus. Most sera from 24 genetically related chickens reared in contaminated surroundings, and showing a high incidence of lymphomatosis, did show the neutralizing antibodies.

Suggestive, though not entirely conclusive, evidence of a relationship between lymphomatosis and Rous sarcoma viruses was obtained from experiments designed to test the hypothesis that the two agents possess common antigens.

The results are discussed from the point of view of the origin of tumor viruses, and also from the point of view of a possible derivation of sarcoma viruses from the viruses of lymphomatosis through a process of variation.

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Studies on the Metabolism of 2-Benzoylaminofluorene-9-C^{14} and 2-Acetylaminofluorene-9-C^{14} in the Rat*†

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The deacetylation of the carcinogen 2-acetylaminofluorene (AAF) by the rat was demonstrated by Morris, Weisburger, and Weisburger (12) and seemed to indicate that 2-aminofluorene (AF) is a major product of the metabolism of AAF. AF has been shown to be almost as carcinogenic for the rat as AAF (11). It appeared possible, therefore, that AF might be the principal agent responsible for the initiation of neoplastic growth by this class of foreign amines and that only compounds which yield AF during metabolism would be carcinogenic. This view, first expressed by Bielschowsky (3), was supported by experiments of Ray and Argus (14) who studied the metabolism of S^{35}-labeled *p*-toluenesulfonamidofluorene (TSAF), which is not carcinogenic. Only 0.5 per cent of the ingested compound appeared in the urine as diazotizable material, stated to be AF; this indicated very weak hydrolysis *in vivo*. In addition, there were differences in the distribution and excretion of the administered radioactivity from AAF and TSAF.

In the light of these experiments, it appeared of interest to investigate the metabolism of 2-benzoylaminofluorene (BAF) which had been shown to be only weakly, if at all, carcinogenic.[1] If the view expressed above concerning the role of AF in the initiation of neoplastic growth is correct, then the low carcinogenicity of BAF might be the result of the absence of or a very slow rate of debenzoylation of the compound *in vivo*. It might be anticipated that little or no AF would be excreted in this instance. Consequently, urine, which had been shown to be the major route of excretion of diazotizable material after administration of AF (6), was extracted with diethyl ether at various pH following administration of labeled BAF and AAF, and the appropriate fraction was examined for the presence of AF. No AF was found after injection of BAF and only a trace after administration of AAF, but the fractionation yielded information concerning the properties of other, as yet unidentified, metabolites. In addition to these experiments, the tissue distribution and the rate of excretion of BAF were studied and compared to those of AAF.

Preliminary *in vitro* experiments showed that AF could not be quantitatively recovered by treatment of BAF with acid or alkali. Consequently, the method of diazotization and coupling with R-salt which has been used in studies on the metabolism of AF and AAF (6, 13, 16) could not be applied. BAF and AAF were therefore synthesized with C^{14} in the 9 position, essentially by the method of Ray and Geiser (15), and the radioactivity present in tissues and excreta after administration of the labeled compounds was measured. Since the study on the metabolism of TSAF was complicated by its poor absorption from the gastrointestinal tract, BAF and AAF were injected intramuscularly into rats. The animals were sacrificed 96 hours after administration. It was felt that this procedure allowed sufficient time for metabolic alteration of the administered material.

MATERIALS AND METHODS

Animals.—Male rats of the Sprague-Dawley strain, weighing 100–300 gm., were used. Water and food were allowed freely during the experiments.

Preparation of labeled compounds.—2-Benzoylaminofluorene-9-C^{14}: The intermediate 2-aminofluorene-9-C^{14} was prepared by the method of Ray and Geiser (15) with modifications. The carbonation of the Grignard reagent, derived from 1.4 gm. of 2-iodobiphenyl with 65.8 mg. of $BaC^{14}O_3$ (5 mc.) and 920 mg. of $BaCO_3$, was carried out at $-10°$ C. instead of $-70°$ C. Under these conditions, 500 mg. of diphenyl-2-carboxylic acid-C^{14}, m.p. 111°–112° C.,[2] was obtained (60 per cent yield). After ring closure of the carboxylic acid, fluorenone-9-C^{14} was reduced by the modified Clemmensen method, with

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† Reviewed in the Veterans Administration and published with the approval of the Chief Medical Director. The statements and conclusions published by the authors are the result of their own study and do not necessarily reflect the opinion or policy of the Veterans Administration.

[1] Personal communication of Dr. F. E. Ray.

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[2] All melting points are uncorrected.

addition of 3 ml. of glacial acetic acid, which aids in the solution of the fluorenone. The yield of fluorene-9-C^{14}, m.p. 110° C., was 350 mg. (90 per cent of theory). Ray and Geiser (15) reported a yield of 75 per cent in the absence of glacial acetic acid. Two hundred and forty mg. of 2-aminofluorene-9-C^{14}, m.p. 126° C., having a specific activity of 6.3 μc/mg, was obtained by nitration and reduction of fluorene-9-C^{14}. Eighty mg. of benzoyl chloride was added to 50 mg. of 2-aminofluorene-9-C^{14} and 50 mg. of unlabeled AF in 2 ml. of hot pyridine. After the reaction mixture had been kept at 100° C. for 5 minutes, it was cooled, diluted with an equal volume of water and made acid by the addition of concentrated hydrochloric acid. The product was collected and washed thoroughly with distilled water. After two recrystallizations from dilute ethanol, 120 mg. (a yield of 86 per cent) of 2-benzoylaminofluorene-9-C^{14} with a specific activity of 2.1 μc/mg and a m.p. 219°–220° C., were obtained. Bachmann and Barton (1), who first prepared 2-benzoylaminofluorene, reported a melting point of 215°–216° C. and the correct nitrogen analysis. Ray[3] has prepared the same compound with a melting point of 221° C. Nonradioactive 2-benzoylaminofluorene, prepared under identical conditions and with the same melting point, was tested for the presence of diazotizable amino groups by the modified method of Westfall, which permits the detection of 1.0 μg. of aminofluorene (6). The test was negative with 17.5 mg. of 2-benzoylaminofluorene.

2-Acetylaminofluorene-9-C^{14}: This compound, m.p. 198° C., was synthesized by the acetylation of 2-AF-9-C^{14}, prepared by the method outlined (15).

Administration of labeled compounds.—The radioactive compounds were dissolved in 2–3 ml. of warm propylene glycol. One to 1.5 ml. of the solution was injected into the muscles of each thigh. The syringe and needle were rinsed with acetone, and the washings made up to volume. The radioactivity of aliquots of this solution was measured. The amount of compound actually injected, as reported in the tables, was the difference between the material dissolved in propylene glycol and that recovered in the washings.

Preparation of tissues and fractionation of urine.—Following injection, the animals were put in all-glass metabolism cages, which permitted the separate collection of urine, feces, and respiratory CO_2. Air, which was freed of carbon dioxide by being passed through two towers of soda lime and ascarite, was drawn through the cage at a rate of approximately 300 ml/minute. The expired carbon dioxide was absorbed in 150 ml. of 30 per cent sodium hydroxide solution, which was changed every 8 hours. Barium carbonate was precipitated from duplicate aliquots by the addition of ammonium chloride and barium chloride (4). The carbonate was collected and counted as described below.

Urine was collected at 24-hour intervals in receivers which contained a few drops of concentrated hydrochloric acid and was made up to a suitable volume with distilled water. Duplicate aliquots were dried *in vacuo* over phosphoric anhydride prior to combustion.

After the experimental period, the animals were anesthetized with ether, and 5–10 ml. of blood was taken by heart puncture. The individual organs were excised, blotted with filter paper, and dried *in vacuo* over phosphoric anhydride. The injection site (femur and surrounding muscles) and the carcass were autoclaved for 90 minutes at 15 lbs/in^2 and then homogenized with distilled water in a Waring Blendor. To insure uniform distribution of the radioactivity, aliquots of these mixtures were rehomogenized in an all-glass homogenizer. The samples were then dried as described. Prior to combustion, the tissues were ground in a mortar and weighed.

The urine was adjusted to pH 2–3 with concentrated hydrochloric or sulfuric acid and extracted in a separatory funnel with several changes of equal volumes of peroxide-free diethyl ether. Completeness of the extraction was gauged by radioactivity measurements of the successive ether layers. Extraction was discontinued when the last extract contained less than 0.2 per cent of the radioactivity in the aqueous phase. Usually four or five extractions were necessary. Although some of the radioactivity in the ether could theoretically be due to the slight miscibility of the two solvents, the results obtained on the extraction of urine by ether at pH 7 prior to boiling with acid, shown below in Chart 2, indicate that such contamination was negligible. The same procedure was applied to the urine at pH 7.0–7.5 and 11–12. The separate extracts and the aqueous layer were made up to appropriate volumes and duplicate aliquots of each fraction evaporated to dryness prior to combustion. Following ether extraction, sufficient concentrated sulfuric acid was added to the urine to give a concentration of 3 N, and the urine was refluxed for 18 hours. The cooled solution was re-extracted with diethyl ether at pH 2–3, 7.0–7.5, and 11–12, as described. Duplicate aliquots of the various fractions were evaporated to dryness prior to combustion. Control experiments with radioactive AF and BAF showed that these compounds were quantitatively extracted from urine at pH 11–12 by diethyl ether.

Extraction of carcass and injection site with organic solvents.—The dry, powdered tissues were extracted successively in a Soxhlet apparatus with several solvents. Aliquots of the tissues were removed initially and, after each change of solvent, weighed and combusted. The radioactivity of the resulting barium carbonate precipitate was measured as described below.

Carrier experiments.—The isolation of AF and BAF from the urine, feces, and carcass of rats dosed with BAF-9-C^{14} was carried out as follows. The ether extract of the alkaline urine (pH 11–12) was evaporated to dryness.[4] Eighty mg. of unlabeled AF, m.p. 127°–128° C., in 10 ml. of ethylacetate was added to the residue, and dry hydrogen chloride was passed into the solution for 1 hour. AF hydrochloride, which precipitated, was separated by centrifugation. The free amine was obtained by treatment with dilute ammonium hydroxide and was crystallized from dilute ethanol to constant specific radioactivity. One hundred mg. of unlabeled BAF, m.p. 219° C., in 22 ml. of hot ethanol was added to the ethylacetate solution following removal of the amine salt. The boiling solution was treated with Norite, filtered, and cooled. BAF, which precipitated on standing, was crystallized to constant specific radioactivity from dilute ethanol.

The ground feces were extracted continuously with boiling acetone for 72 hours. Two hundred mg. of unlabeled BAF was then added to the acetone extract. The acetone was evaporated, the residue dissolved in boiling ethanol, treated with Norite, and filtered. The hot filtrate was diluted

[3] Personal communication of Dr. F. E. Ray.

[4] All carrier experiments involving the urine were performed before boiling with sulfuric acid.

with water to incipient precipitation and allowed to cool. The solid was recrystallized from dilute ethanol to constant specific radioactivity.

Eighty mg. of unlabeled BAF was added to an acetone extract of carcass (Rats 1 and 2) which had been previously extracted with ether. The recovered BAF was treated as described above and crystallized to constant specific radioactivity.

The isolation of AF and AAF from the urine of the rat dosed with 2-AAF-9-C^{14} was carried out essentially as described above for BAF. One hundred and fifty mg. of unlabeled AF and 157 mg. of unlabeled AAF, m.p. 198° C., were used as carrier compounds.

The purity of BAF and AAF which had been crystallized to constant specific radioactivity was confirmed by the constant solubility test (7) employing dilute ethanol as the liquid phase. AF obtained from the urine of the rat injected with AAF-9-C^{14} was converted to AAF with no change of the specific radioactivity, indicating that the isolated material was pure.

Radioactivity measurements.—Samples which weighed from 1 to 5 mg. were combusted in duplicate by an adaptation of the procedure of Lindenbaum, Schubert, and Armstrong (9) to the micro scale. The tissues were weighed to 0.1 mg. on an analytical balance, the labeled compounds to 0.01 mg. on a semimicrobalance. The barium carbonate precipitates were collected on filter paper and counted in a windowless gas-flow counter. The counting times were such that the standard error did not exceed 5 per cent, except when the radioactivity of the sample was less than 25 per cent above background. In these cases, the standard error was 10 per cent. Samples having counting rates of less than 5 per cent above background (≤ 1 c.p.m.) were considered inactive. The radioactivities were corrected for self-absorption.

TABLE 1

THE DISTRIBUTION OF THE ADMINISTERED RADIOACTIVITY IN THE TISSUES AND EXCRETA OF THE RAT AFTER INTRAMUSCULAR INJECTION OF C^{14}-LABELED BAF AND AAF

	RATS NOS. 1 AND 2 (2-BAF-9-C^{14})*		RAT NO. 3 (2-BAF-9-C^{14})†		RAT NO. 4 (2-AAF-9-C^{14})‡	
	Specific activity cpm/mg	Per cent of dose recovered	Specific activity cpm/mg	Per cent of dose recovered	Specific activity cpm/mg	Per cent of dose recovered
Expired CO_2				<0.06		<0.06
Urine		8.41		15.9		47.9
Feces	65.1	11.6#	78.4	82.5#	95.8	39.5#
Bladder					0.0	0.0
Blood§	1.6	0.17			5.1	0.80
Brain	0.9	0.01			0.0	0.0
Caecum with contents	45.4	1.43			17.9	0.68
Carcass	32.9	59.0	1.1	1.1	0.4‖	0.80
Colon					4.0	0.07
Heart					1.6	0.01
Injection site	935	20.5	45.7	5.84	17.7	4.48
Kidneys	2.3	0.04			4.4	0.08
Liver	3.9	0.37			9.2	0.89
Lungs	3.5	0.04			1.7	0.02
S. Intestine with contents	16.0	0.89			9.4	0.60
Spleen	1.3	0.01			1.6	0.01
Stomach with contents	3.8	0.13			6.7	0.16
Thymus					0.0	0.0
Total		103		105		95

* Rats Nos. 1 and 2 received 15.95 mg. of labeled BAF (6,790,000 c.p.m.). The rats were sacrificed after 4 days, and the tissues and excreta were pooled.

† Rat No. 3 received 4.92 mg. of labeled BAF (2,090,000 c.p.m.). The respiratory air was collected for 4 days and the rat was sacrificed after 11 days.

‡ Rat No. 4 received 7.35 mg. of labeled AAF (1,966,000 c.p.m.). The rat was sacrificed after 4 days.

§ The total radioactivity of the blood was calculated on the basis of 6.7 ml of blood/100 gm of body weight (5).

\# The contents of the colon were added to the feces.

‖ The radioactivity of this sample was less than 5 per cent above the background (<1 c.p.m.).

RESULTS AND DISCUSSION

The distribution of the administered radioactivity after intramuscular injection of labeled BAF and AAF is shown in Table 1. It will be seen that, 96 hours after administration of BAF, as much as 79 per cent of the radioactivity was present in the body of the rat, 59 per cent having been found in the carcass and 20 per cent at the site of injection. Only 20 per cent was excreted in the urine and feces. The retention of relatively large quantities of radioactivity in the carcass and the injection site after administration of BAF raised the possi-

bility that some of the labeled material might have been chemically bound to the tissues. Since *p*-dimethylaminoazobenzene and 3,4-benzpyrene which are bound to liver and epidermal proteins could not be removed by extraction with organic solvents (8, 10), the carcasses and the injection sites from the rats treated with BAF were continuously extracted with a number of organic solvents. Table 2 shows that 97 and 99 per cent of the radioactivity of the carcass and of the injection site were soluble in organic solvents and were, therefore, presumably not firmly bound to the tissues. Although BAF was isolated from the acetone extract of the carcass, it accounted only for 45 per cent of the radioactivity of the extract (Table 3). This indicates that, in addition to the administered compound, a metabolite or metabolites of BAF contributed to the radioactivity of the carcass. As shown in Table 1, the radioactivity of BAF in the carcass and at the injection site was eliminated during the subsequent 7 days, since 98 per cent could be accounted for in the excreta 11 days after injection. Inasmuch as 83 per cent of the radioactivity was found in the feces and only 16 per cent in the urine at that time, it appears that BAF was excreted predominantly through the bile and gastrointestinal tract. The specific activities of liver, kidneys, lungs, and spleen 96 hours after injection of BAF indicated a relatively even distribution of the labeled material throughout the internal organs. Less than 0.06 per cent of the administered radioactivity was found in the expired air after injection of BAF or AAF. This amount of radioactivity represented the lower limit of detection. These findings confirm the conclusion of Morris, Weisburger, and Weisburger (12) that carbon-9 of fluorene is not oxidized to carbon dioxide *in vivo*.

The distribution of labeled AAF differed strikingly from that of BAF. In contrast to BAF, the

TABLE 2

EXTRACTION OF RADIOACTIVITY FROM CARCASS AND INJECTION SITE BY ORGANIC SOLVENTS

Solvent	Time of extraction (Hours)	BAF Carcass* cpm/mg	BAF Injection site† cpm/mg	AAF Injection site‡ cpm/mg
None	0	33	935	18
Diethyl ether	72	13	214	8
Acetone	48	6	70	5
Ethanol	48	1	5	3
Benzene	48		3	2

* 1.11 Gm. of tissue from Rats Nos. 1 and 2 injected with 2-BAF-9-C^{14} was extracted.
† 1.49 Gm. of tissue from Rat Nos. 1 and 2 injected with 2-BAF-9-C^{14} was extracted.
‡ 0.33 Gm. of tissue from Rat No. 4 injected with 2-AAF-9-C^{14} was extracted.

TABLE 3

IDENTIFICATION OF METABOLITES BY CARRIER EXPERIMENTS

Compound administered	Compound isolated	Carrier added to	Specific radioactivity of isolated compound (cpm/mg) 1*	2*	3*	4*	Per cent of radioactivity in extract identified	Per cent of injected compound found
2-BAF	2-AF	Ether extract of urine†	8.4	0‡			0.0	0.00
"	2-BAF	Ether extract of urine†	78	49	48	53	40	0.08
"	2-BAF	Acetone extract of feces§	97	64	53	51	2.3	0.10
"	2-BAF	Acetone extract of carcass#	45	38		42	45	4.70
2-AAF	2-AF	Ether extract of urine‖	16	10	7.3	5.9**	0.2	0.06
"	2-AAF	Ether extract of urine‖	171	151	126	127††	7.4	0.98

* The compounds were crystallized from dilute ethanol as described in the text.
† Ether extract of alkaline urine from Rats Nos. 1 and 2 injected with 2-BAF-9-C^{14}.
‡ The radioactivity of three successive samples was less than 5 per cent above background (<1 c.p.m.).
§ Acetone extract of feces of Rats Nos. 1 and 2 injected with 2-BAF-9-C^{14}.
\# The carcass of Rats Nos. 1 and 2 had been previously extracted with ethyl ether for 72 hours as described in the text.
‖ Ether extract of alkaline urine of Rat No. 4 injected with 2-AAF-9-C^{14}.
** This value is the specific radioactivity of AAF obtained by acetylation of AF from crystallization No. 3.
†† Dilute methanol was the solvent for this crystallization.

radioactivity of AAF was rapidly excreted, 48 per cent having been found in the urine and 40 per cent in the feces 96 hours after administration. Approximately 4.5 per cent of the administered radioactivity was retained at the site of injection, 89 per cent of which could be removed by boiling organic solvents (Table 2). Since in this instance 11 per cent of the radioactivity could not be extracted from the tissue, the possibility exists that small amounts of AAF or of derivatives were combined with tissue constituents. It will be noted that the specific activity of the liver was approximately twice as great after injection of AAF as after administration of BAF. The concentration of radioactivity in the liver after injection of AAF is of interest, since this organ is one of the chief sites of tumor induction by AAF (11).

The differences in the rates of elimination of the two compounds are reflected in the quantities of labeled material which were excreted per day in the urine (Chart 1). Following the injection of labeled AAF, the radioactivity was excreted ap-

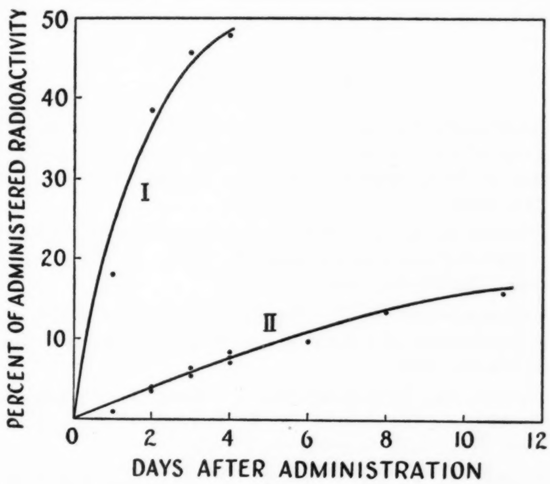

CHART 1.—Urinary excretion of radioactivity following intramuscular injection of 2-AAF-9-C^{14} (I) and 2-BAF-9-C^{14} (II).

proximately 10 times faster than after administration of a comparable quantity of labeled BAF. As will be shown below, 70–90 per cent of the urinary radioactivity was associated with water-soluble products, whereas the administered compounds are insoluble in water. It appears, therefore, that the metabolic reactions, which rendered the injected compounds water-soluble, proceeded at a faster rate in the case of AAF. This suggests that AAF is more reactive *in vivo* than is BAF and that the lower chemical reactivity of BAF may account for the longer retention of the compound in the rat. The rapid excretion of AAF is comparable to rates which have been observed for other labeled carcinogens. Thus, 83 per cent of the radioactivity of intravenously administered 3,4-benzpyrene-5-C^{14} was eliminated in 24 hours (8) and 70 per cent of the nitrogen of fed N^{15}-labeled *p*-dimethylaminoazobenzene was recovered in the urine of the rat in 72 hours (2). The high rate of excretion of AAF may also help to explain why AAF must be fed continuously for several months before tumors appear.

The results of the fractionation of urine, which had been collected for 96 hours following administration of the labeled compounds, are shown in

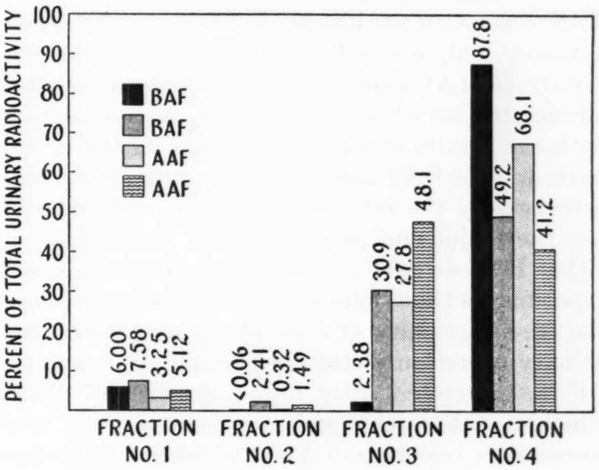

CHART 2.—Partition of urinary radioactivity with ether. Fraction No. 1 represents the radioactivity extractable at pH 2-3, fraction No. 2 at pH 7.0–7.5, fraction No. 3 at pH 11–12. Fraction No. 4 shows the water-soluble radioactivity. The first and third columns of each fraction indicate the radioactivity before boiling with sulfuric acid, the second and fourth columns represent the radioactivity after boiling with sulfuric acid. The numbers on top of the columns are the per cent of the urinary radioactivity in the fractions.

Chart 2. The major portion of the radioactivity, 88 per cent, was not extractable by ether at acid, basic, or neutral pH from the urine of rats which had been dosed with labeled BAF. Likewise, 69 per cent of the radioactivity could not be extracted from the urine of the rat which had been injected with labeled AAF. Additional radioactivity could be removed by ether extraction after prolonged boiling of this fraction with sulfuric acid. The water-soluble radioactivity in the urine of rats treated with labeled BAF decreased from 88 per cent to 49 per cent. A similar decrease in the water-soluble radioactivity occurred when the urine of the rat injected with labeled AAF was hydrolyzed. It seems likely that the water-soluble fraction of the urinary radioactivity was associated with glucuronides and/or ethereal sulfates which may have been formed from hydroxylated BAF and AAF and which were partially cleaved by treatment of

the urine with hot acid. The radioactivity which was found in ether extracts from acid or alkaline urine indicates the presence of other, as yet unidentified, metabolites. Unchanged BAF accounted for 40 per cent, and AAF and a trace of AF accounted for 7.6 per cent of the radioactivity extractable by ether from alkaline urine of rats injected with BAF and AAF, respectively (Table 3).

The identification of the compounds which were isolated by carrier experiments is summarized in Table 3. No AF was detected in the urine after injection of BAF. Only 0.2 per cent of administered BAF was found unchanged in the urine and feces. Likewise, only a small quantity of AAF and a minute trace of AF were recovered from the urine after administration of AAF. The data, in conjunction with the results of the fractionation of the urine, indicate that BAF and AAF were extensively metabolized by the rat and that AF is undoubtedly not the major end product of the metabolism of either BAF or AAF. It appears from the present experiments that there is no relationship between the carcinogenicity of derivatives of AF and the urinary excretion of the free amine. Although no AF was excreted after administration of BAF, which is not carcinogenic, only little more was found after injection of AAF, which is highly carcinogenic. The possibility remains that AF is formed from AAF in the tissues, but is rapidly further metabolized. Since the major portion of the excretory products, after administration of derivatives of AF, is not AF, it is not clear how much of the diazotizable material found in the urine after administration of AAF or TSAF (13, 14) was actually AF. In the light of the present and previous findings (6), it would appear that the method of diazotization and coupling with R-salt is not specific enough for the quantitative measurement of AF in the urine or other biological materials.

SUMMARY

1. The metabolism of 2-benzoylaminofluorene-9-C^{14} and 2-acetylaminofluorene-9-C^{14} was studied in the rat after intramuscular injection. The administered radioactivity was recovered quantitatively from the tissues and excreta.

2. Approximately 90 per cent of the radioactivity of labeled AAF was excreted in 96 hours, while only 20 per cent of the radioactivity of labeled BAF was found in the excreta. Labeled material was excreted in the urine approximately 10 times faster after injection of AAF than after administration of BAF. The data indicate that AAF is more reactive *in vivo* than is BAF.

3. Fractionation of the urine with ether at various pH indicated that the major portion of the radioactivity was associated with water-soluble compounds which may be conjugation products. Treatment of the urine with hot sulfuric acid decreased the quantities of water-soluble radioactivity, indicating partial hydrolysis.

4. No AF was recovered from the urine after injection of labeled BAF and only a trace after administration of labeled AAF. AF is not, therefore, considered to be a major end product of the metabolism of BAF or AAF. Since no appreciable difference in the recovery of AF after administration of BAF or AAF was found, there appears to be no relationship between the carcinogenicity of derivatives of AF and the excretion of the free amine.

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The Transplantation of Human Brain Tumors to the Brains of Laboratory Animals*

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The successful transplantation of human brain tumors to the eye of the guinea pig has been described (5, 6), and the ability of such tumors to survive transfer to the brain has also been noted (1). Since the latter report, a group of human brain tumors, representing various histological types, have been tested with respect to heterotransplantability in the brain; and one tumor, a glioblastoma multiforme, has been carried by serial brain transfer in several animal species. The results of this study form the substance of the present report.

MATERIALS AND METHODS

The technic of brain transfer has been described in detail (1). The tumors were either transplanted immediately after operation or allowed to stand in sterile containers in the refrigerator at 5° C. for variable periods of time. The longest interval between operation and transfer was 90 hours, and in this instance takes occurred in a large percentage of the animals used.

The distortion of structural relations incident to the surgical removal of brain tumors usually makes a gross differentiation of elements extremely difficult. Frozen sections were employed to identify tissues; but, despite careful selection of material, fixation and microscopic study of adjacent areas suggested that in many cases the fragments used for transfer consisted of necrotic tissue or normal brain rather than of tumor tissue. Accordingly, to insure an adequate test of the transplantability of the tumor, twenty animals, consisting of eight guinea pigs and twelve DBA mice, were used in each experiment; and the fact that takes occurred in two or more animals of each species in the case of all transplantable tumors indicates that the numbers employed were sufficient. It is apparent, however, in view of the uncertain composition of the transplanted material, that the proportion of takes and nontakes obtained on transfer may be no more than a measure of the cellularity of the tumor or the care exercised in the selection of fragments and, as such, cannot be used as an index of the biological constitution of the growth. For this reason, the successful transfer of a tumor is recorded in the accompanying table by a plus (+) sign, and no attempt is made to characterize transplantability in quantitative terms.

Comparable difficulties are not encountered in obtaining tissue for serial brain transfer, for the large tumor is free of necrosis, readily apparent on gross section, and can be dissected clear of the surrounding brain.

RESULTS

Primary transfers.—The results of the transfer of 22 different human brain tumors are shown in Table 1. The series includes two astrocytomas, three ependymomas, four meningiomas, one oligodendroglioma, one choroid papilloma, and eleven glioblastomas. The only transplantable tumors in the group were glioblastomas, and eight of the eleven were successfully transplanted.

The behavior of the animals bearing the transplantable tumors was similar in all cases. As a rule, neurological signs were not observed until the growing transplant occupied the greater part of a cerebral hemisphere, and their appearance was followed by death within a few hours. The interval between transfer and death in such cases averaged 4 months. In rare instances, death, following signs of increased intracranial pressure, occurred during the 2d month, but the tumors found at autopsy were relatively small, and the short survival did not appear to be related to a more rapid growth rate.

The rate of growth was generally the same in mice and guinea pigs, but in one instance (Case No. 16) growth was more rapid in the mouse, and

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large tumors were found in animals killed during the 7th week. The growing transplants were detectable on gross examination of the brain in all animals killed a month after transfer, but at earlier intervals histological section was required.

Histologically, the transplants resembled the parent tumors but invariably showed an increased content of spongioblasts. Giant cells, palisading, and capillary endothelial proliferation were common features (Figs. 1–17).

In the guinea pig, the tumors were well circumscribed and surrounded by compressed brain tissue, suggesting that growth was predominantly expansive in character. In the mouse, on the other hand, growth was invasive from the beginning. There was no clear line of demarcation between the transplant and surrounding brain, and irregular tongues of loosely arranged tumor cells extended from the main mass into the neighboring tissue with little apparent damage to nerve cells.

Serial transfers.—Serial transfer of the tumors was successful whenever attempted, and several were carried for a number of generations with no notable change in general behavior. It seems probable that all the tumors could have been maintained in this manner; but such a procedure was not attempted, and the material utilized in further studies of transplantable brain tumors was derived from a human glioblastoma previously carried in the guinea pig's eye.

This tumor (HC305) was first transferred from man to guinea pig by Dr. Edward Krementz in January, 1948. Tissue from an anterior chamber growth was transplanted to the brain in November, 1950, and has been carried to date by serial brain transfer. Its behavior has been studied in the guinea pig, mouse, rabbit, and rat, and attempts have been made to transplant it to different bodily regions in these species. At present, the tumor has been carried for thirteen consecutive passages in the guinea pig and mouse and for five passages in the rabbit and rat.

Takes occur in all animals of the various species, and growth continues until death. The rate of growth is more rapid in the rabbit and rat than in the guinea pig and mouse. The tumors attain a sufficient size to kill guinea pigs early in the 4th month; mice generally die a few weeks earlier, while rabbits and rats rarely survive beyond the 2d month. However, the rate is much more consistent in the guinea pig, and wide fluctuations characterize growth in different animals of the other species.

Histologically, the transplants show all the classical features of glioblastoma multiforme (Figs. 18–31). Growth in the guinea pig, rabbit, and rat is largely expansive in character, whereas diffuse, invasive tumors are characteristic of the mouse. Metastasis within or without the brain substance has never been observed.

Transfer from the brain to other bodily sites.—Numerous attempts have been made to transfer

TABLE 1

THE RESULTS OF TRANSFER OF A SERIES OF HUMAN BRAIN TUMORS TO THE BRAINS OF GUINEA PIGS AND MICE

Case no.	Patient	Tumor	Date of transfer	Result	Present status of patient
1	R. E.	Astrocytoma	6/24/50	−	Died 1/14/51
2	H. S.	Astrocytoma	6/28/50	−	Died 12/14/50
3	M. K.	Ependymoma	5/16/50	−	Died 4/21/51
4	A. M.	Ependymoma	7/24/50	−	Died 3/16/51
5	C. S.	Ependymoma	10/ 3/50	−	Died 3/12/51
6	T. C.	Oligodendroglioma	1/10/52	−	Alive
7	P. W.	Choroid papilloma	10/ 2/50	−	Died 9/20/51
8	S. G.	Meningioma	8/12/50	−	Alive
9	L. P.	Meningioma	8/15/50	−	Alive
10	J. M.	Meningioma	10/13/50	−	Died 11/ 3/50
11	J. S.	Meningioma	11/ 7/51	−	Died 11/11/51
12	A. R.	Glioblastoma	8/20/50	+	Died 3/13/51
13	J. M.	Glioblastoma	9/ 7/50	+	Died 10/ 8/50
14	L. S.	Glioblastoma	9/19/50	+	Died 11/ 7/51
15	L. B.	Glioblastoma	10/20/50	+	Died 11/10/50
16	C. J.	Glioblastoma	11/ 1/50	+	Died 11/ 2/50
17	S. R.	Glioblastoma	11/29/50	−	Died 11/29/50
18	D. W.	Glioblastoma	11/30/50	+	Died 12/ 1/50
19	G. D.	Glioblastoma	4/13/51	+	Died 4/14/51
20	P. P.	Glioblastoma	4/27/51	+	Died 11/28/51
21	G. W.	Glioblastoma	5/24/51	−	Died 5/25/51
22	J. M.	Glioblastoma	11/ 2/51	−	Died 2/13/52

the tumors from the brain to other bodily regions utilizing different technics as well as recipient hosts of different constitutional status. Such investigations were undertaken in an effort to clarify the factors concerned in the failure of the glioblastoma to metastasize. It should be emphasized in this connection that this tumor and the medulloblastoma represent the sole exceptions so far encountered to the rule that only metastasizable tumors possess the ability to grow on heterologous transfer (3). The existence of a blood-brain barrier has been invoked in an attempt to explain the failure of brain tumors to metastasize, but the observation that other tumors, such as the Brown-Pearce carcinoma, metastasize throughout the body from brain transplants constitutes strong evidence against a barrier of this nature and suggests that the factors concerned reside in the tumor itself rather than in the site of origin. As part of an investigation designed to test this suggestion, experiments were undertaken to determine whether or not glioblastoma tissue could be grown in other bodily regions.

Fresh tumor tissue obtained from growing brain transplants was transferred to different sites including the testicle, kidney, lung, spleen, liver, muscle, and subcutaneous space of guinea pigs, mice, rats, and rabbits. Many of the animals were observed for as long as 5 months, others were killed at intervals for histological study, but in no instance was any indication of growth of the transplant found. Such a result is in contrast with experience with other human tumors. A large number of human tumors have been studied, and, although first-generation passage from man to a heterologous species can be effected only in the eye or brain, second-generation passage from the animal's eye or brain to other bodily sites is generally successful.

The failure to obtain takes suggested the use of a technic sometimes employed to increase the incidence of growth of tissue with poor stroma-inducing abilities (4). This technic is based on the high sensitivity of embryonic tissue to stroma-inducing stimuli, and transplants of embryonic organs are used as recipient sites for the transfer of other tissues. In practice, a two-stage transfer has been found unnecessary, and both the embryonic organ and the tissue in question are transplanted simultaneously. Numerous attempts were made to grow glioblastoma in different regions by this method, but, despite its success with other organs and tissues, no evidence of growth of the brain tumor was ever obtained.

In other experiments, brain transplants of the glioblastoma were transferred to newborn animals and to animals bearing spontaneous tumors in different stages of development. It had been noted previously that animals of the latter type provided a suitable environment for the subcutaneous growth of some heterologous tumors, whereas takes did not occur in normal individuals of the same species. Finally, in a number of cases, the glioblastoma was transplanted directly into spontaneous or transplanted tumors growing in various sites, for in other studies heterologous transfer had been accomplished in this manner. These experiments were repeated with tumor tissue obtained from transplants of different age and of different serial generation, and the recipient animals were subjected to thorough microscopic study; but in no case was growing glioblastoma tissue found.

DISCUSSION

The ability of the human glioblastoma multiforme to survive and to grow in the brain of animals of alien species is in sharp contrast to the incapacity of the benign brain tumors in this direction. No takes followed the transfer of ten benign tumors, while growth was obtained with eight of the eleven glioblastomas tested. The failure of three of the glioblastomas to survive heterotransplantation is not clear but may relate to a technical fault in the selection of fragments for use rather than to the constitution of the tumor itself. No indication of a variation in behavior referable to the tumor was apparent in the clinical histories of the patients concerned. The failure of the meningiomas to survive transfer to the brain was contrary to expectation, for such tumors do survive transfer to the anterior chamber (5, 6). Growth is minimal in the eye, but cellular content and architecture are preserved for long periods of time. Although only four meningiomas were studied, 32 test animals were employed, and the failure in all cases is sufficiently suggestive of a differential environmental requirement to warrant further investigation.

With respect to heterotransplantability, the glioblastomas constitute an apparent exception to other tumors. An investigation of many spontaneous tumors of both human and animal origin has shown the existence of a close relationship between metastasizability and heterotransplantability and has led to the conclusion that only metastasizable tumors are heterotransplantable (2). Accordingly, the logical inference to be drawn from the transplantability of the glioblastoma is that it is a metastasizable tumor, but the most characteristic and well established feature of this growth is

the fact that it does not metastasize. Although the inference and the fact appear to be contradictory, a consideration of the mechanics of metastasis offers a possible solution.

It is apparent that, in order to metastasize, tumor cells must possess the ability to invade vascular walls, to survive transport through the blood stream, and to elicit a stroma from the connective tissues at the site of lodgement. The probability that metastasizable tumor cells differ widely with respect to the latter factor is suggested by vagaries in their behavior in the primary host, as well as by variations in their reaction to transplantation. The hypernephroma is an example. This tumor, like other tissue with a scanty connective tissue content, is a poor stroma-inducer. In the primary host, it may invade and extend along the renal vein for some distance, but, despite the fact that cells must inevitably be washed into the circulation, distant foci of growth may not be found. On transfer to the guinea pig's eye, its stroma-inducing properties are usually too slight to elicit a stroma from the highly sensitive connective tissues of the iris, and its growth resembles that of a tissue culture. In order to obtain a vascular supply in the eye, it is necessary to transplant the tumor cells in association with embryonic connective tissue. The stroma of this tissue, being embryonic in character, persists; vascular connections with the iris are established, and the transplant responds to the minimal stroma-inducing stimuli of the hypernephroma cells and supplies them with a connective tissue scaffolding and a blood supply. The connective tissue of the brain, although present in minimal quantity, appears to be as responsive to stroma-inducing stimuli as does that of embryonic organs, and transfer of the hypernephroma to the brain results in a vascularized growth.

The potentialities of embryonic tissue for the production of stroma have been utilized to obtain subcutaneous growth of such poor stroma-inducers as the adrenal gland, and the technic has made it possible to grow a variety of tissues in this region of unresponsive connective tissue. To the present, however, it has not been possible with this or any other technic attempted to grow brain tissue in any other site than the eye or the brain itself, and it would appear that the stroma-inducing abilities of brain tissue are quantitatively less and qualitatively different from those of other tissues. The points to be emphasized in the present relationship are that normal tissues and tumor tissues vary in their ability to elicit a stroma response, that connective tissues in different bodily sites vary in their responsiveness to stroma-inducing stimuli, and that, while the brain contains the most responsive connective tissue in this respect, its substance constitutes the poorest stroma-inducer so far encountered.

A relationship between such findings and the failure of the glioblastoma to metastasize despite its autonomous nature is suggested. All attempts to transplant the tumor to sites other than the brain and eye were unsuccessful, and a variety of technics were employed. The inability of the tumor to grow in other regions is in line with its natural behavior in the human host, and the suggestion is made that the failure to metastasize is the consequence of this inability: in other words, distant foci of growth do not occur, because the tumor is incapable of growing in distant foci.

Experimental results indicate that the failure to grow is based, in turn, on an incapacity of the glioblastoma cells to elicit a stromal reaction. The glioblastoma, like its parent tissue, the brain, is an exceptionally poor stroma-inducer, and its limited capacities in this direction are only sufficient to stimulate a stromal reaction from the highly responsive connective tissues of the brain and eye. Accordingly, the cells of the brain tumor may gain entrance to the blood stream and be transported as viable units to some distant bodily location, yet fail to grow to a recognizable mass because of the absence of supporting stroma and nutritive blood supply.

In any case, the ability to produce metastases differentiates autonomous tumors of the brain from autonomous tumors of other bodily regions, and an explanation of this differential ability derived from the assumption of a blood-brain barrier is opposed by experimental evidence. On the contrary, investigation indicates that the factors concerned are functions of the tumor rather than of its anatomical site and thus offer an approach to the problem of metastasis based on ascertainable differences in the constitutional composition of brain and bodily tissues.

The ability of glioblastomas to survive transfer to the brains of laboratory animals provides a means for the experimental study of clinical problems associated with brain tumors in man. The conditions pertaining to the natural occurrence of brain tumors are closely approximated by the use of the brain as a transplantation site, and any desired area may be selected for growth of the transplant. Transfer is invariably successful after the first passage, and the inoculated animals offer a uniform material for the investigation of questions concerned with therapy as well as with diagnosis and localization of the lesion.

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Fig. 1.—Section of glioblastoma from Case No. 12. ×160.

Fig. 2.—Section of transplant of tumor shown in Figure 1 growing in a guinea pig's brain. The animal was killed 100 days after transfer. ×160.

Fig. 3.—Section of glioblastoma from Case No. 13. ×160.

Fig. 4.—Section of transplant of tumor shown in Figure 3 growing in the brain of a mouse. The animal was killed 57 days after transfer. ×160.

Fig. 5.—Section of glioblastoma from Case No. 14. ×380.

Fig. 6.—Section of transplant of tumor shown in Figure 5 growing in a guinea pig's brain 30 days after transfer. ×380.

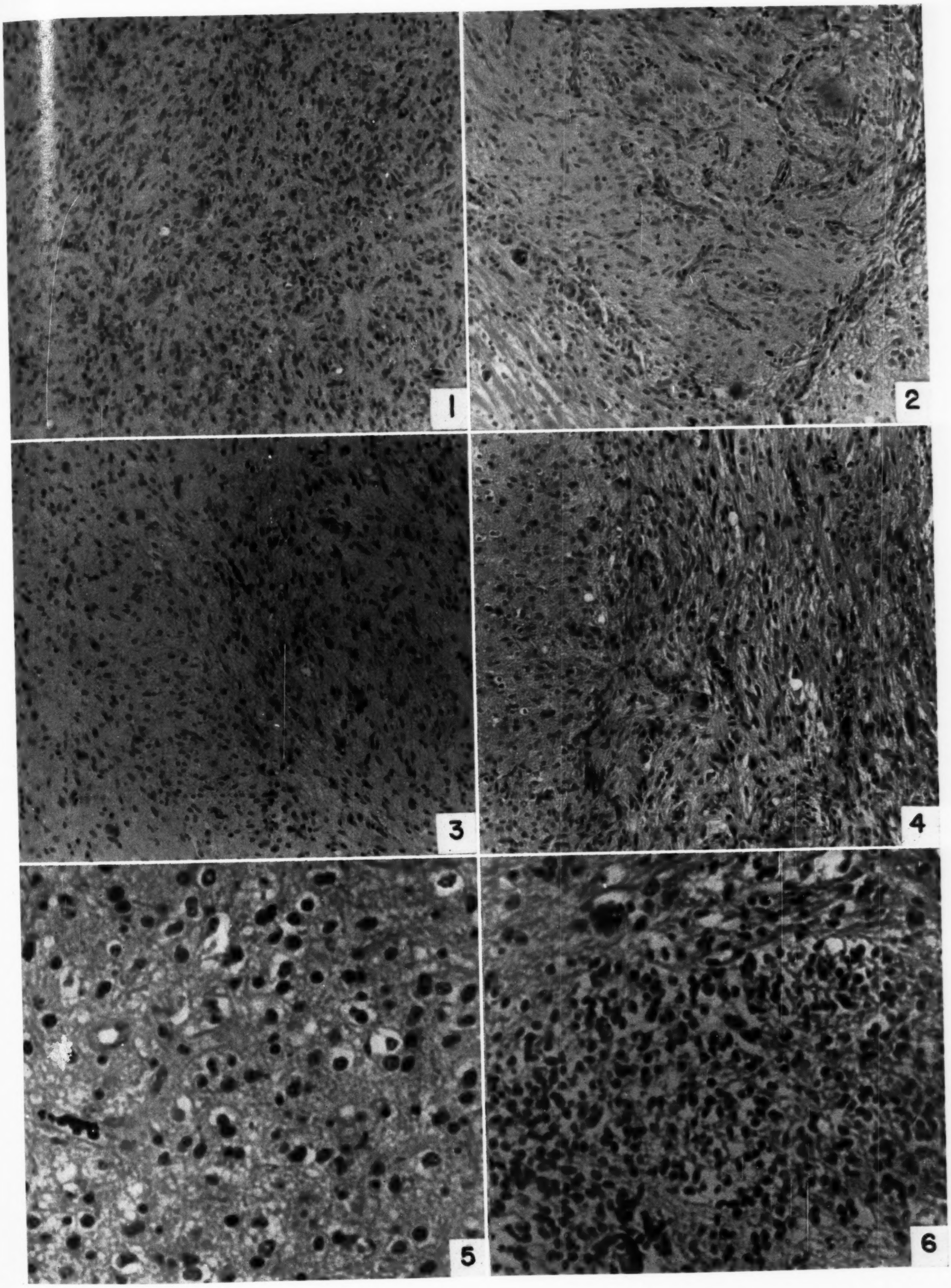
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Fig. 7.—Section of glioblastoma from Case No. 15. ×380.

Fig. 8.—Section of transplant of tumor shown in Figure 7 growing in a guinea pig's brain 87 days after transfer. ×380.

Fig. 9.—Section of glioblastoma from Case No. 16. ×160.

Fig. 10.—Section of transplant of tumor shown in Figure 9 growing in the brain of a mouse 38 days after transfer. ×160.

Fig. 11.—Section of glioblastoma from Case No. 20. ×160.

Fig. 12.—Section of transplant of tumor shown in Figure 11 growing in a guinea pig's brain 60 days after transfer. ×160.

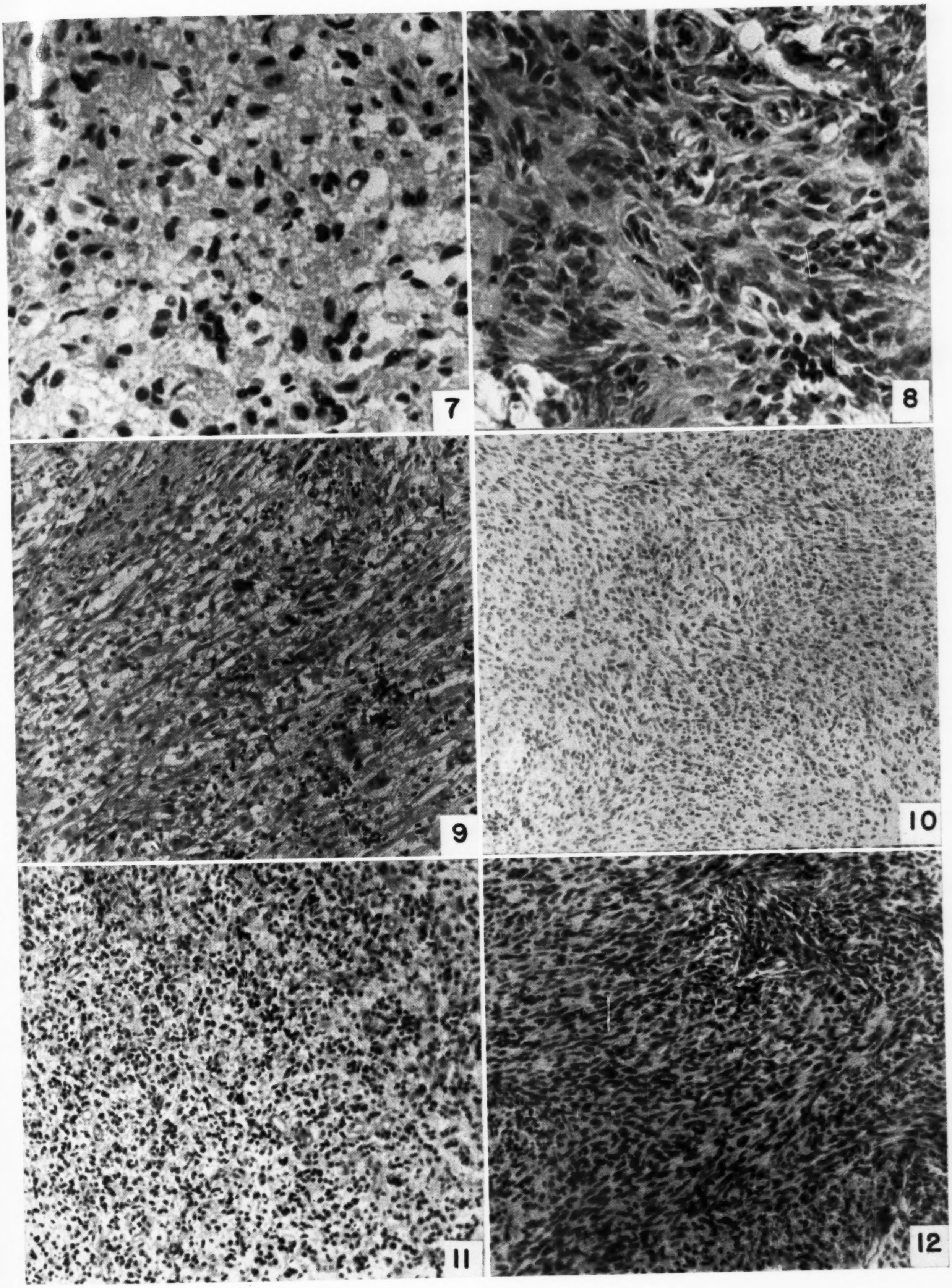
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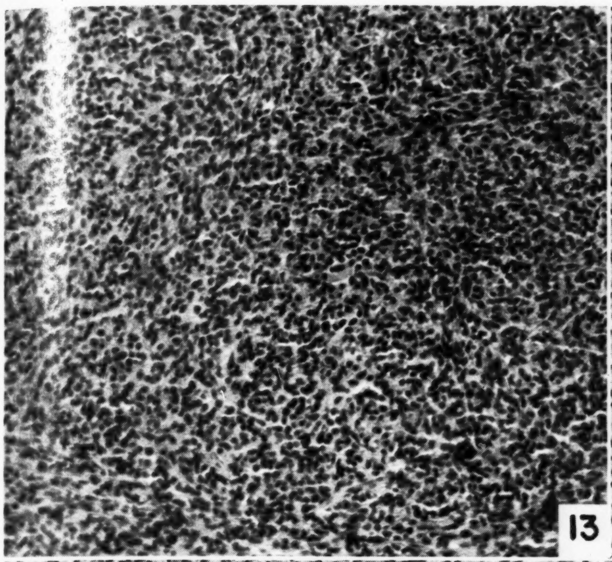
FIG. 13.—Section of transplant of tumor shown in Figure 11 growing in the brain of a mouse 133 days after transfer. ×160.

FIG. 14.—Section of glioblastoma from Case No. 19. ×160.

Fig. 15.—Section of transplant of tumor shown in Figure 14 growing in a guinea pig's brain 134 days after transfer. ×160.

FIG. 16.—Section of transplant of tumor shown in Figure 14 growing in the brain of a mouse 130 days after transfer. ×160.

FIG. 17.—Section of transplant of tumor shown in Figure 14 growing in the brain of a mouse 153 days after transfer. Note preservation of nuclear zone in midst of tumor. ×100.



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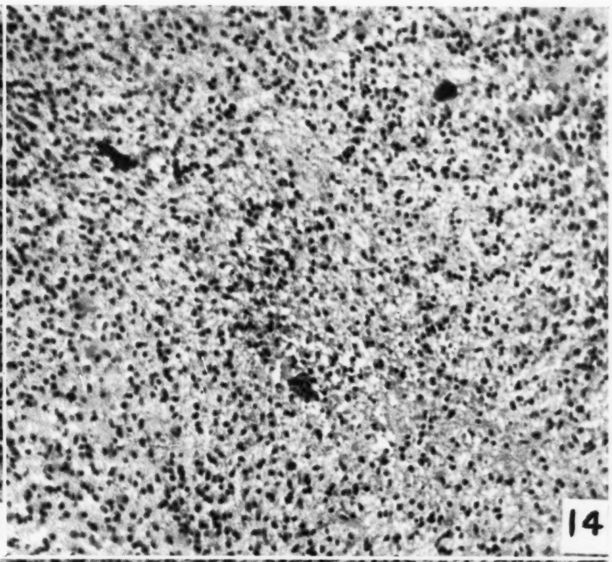

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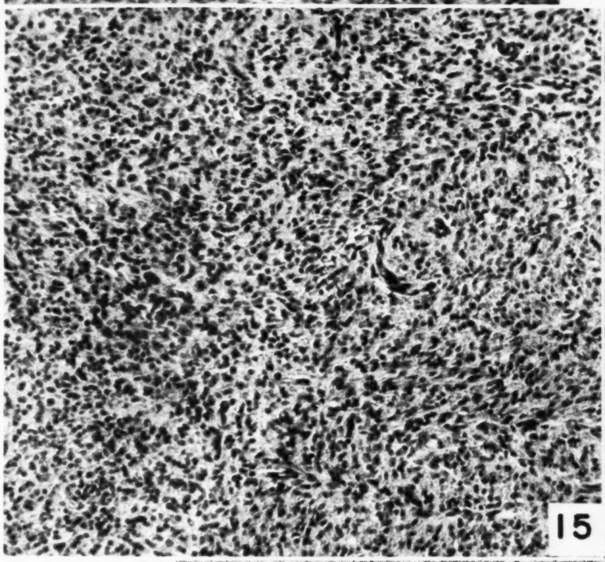

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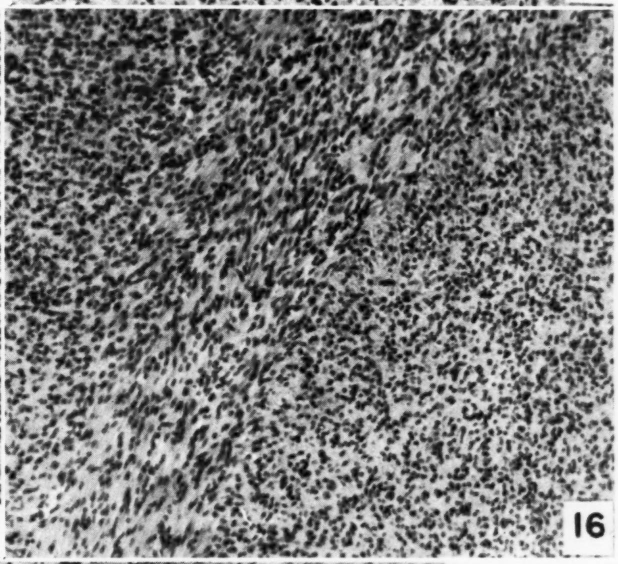

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FIG. 18.—Whole section of guinea pig brain bearing transplant of glioblastoma, HC 305. This was a 7th-generation brain transplant, and the animal was killed 84 days after transfer. $\times 6\frac{1}{2}$.

FIG. 19.—Whole section of mouse brain bearing growing transplant of glioblastoma, HC305. This was a 2d-generation transplant, and the animal was killed 40 days after transfer. $\times 12\frac{1}{2}$.

FIG. 20.—Whole section of rabbit brain bearing growing transplant of glioblastoma, HC305. This was a 1st-generation transplant, and the animal was killed 49 days after transfer. $\times 4\frac{1}{2}$.

FIG. 21.—Whole section of rat brain bearing growing transplant of glioblastoma, HC 305. This was a 2d-generation rat, and the animal was killed 45 days after transfer. $\times 6\frac{1}{2}$.

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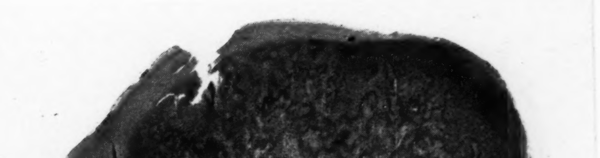

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FIG. 22.—Section of glioblastoma, HC 305, from human patient. ×160.

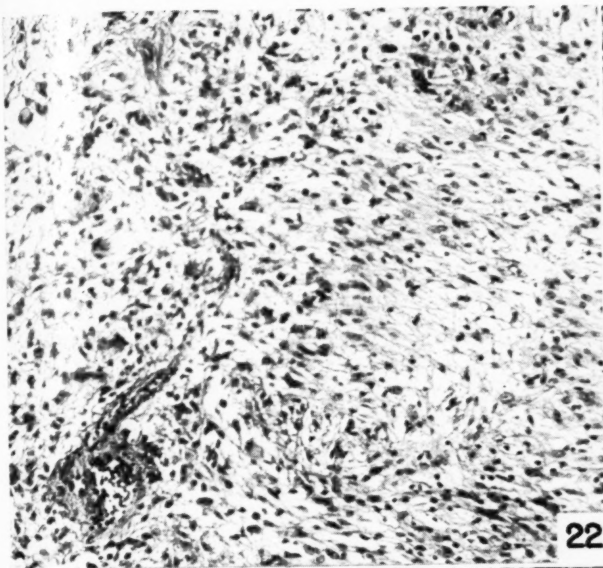
FIG. 23.—Section of transplant of HC 305 in brain of an 8th-generation guinea pig. The animal was killed 54 days after transfer. ×280.

FIG. 24.—Section of transplant of HC 305 in brain of a 4th-generation guinea pig 92 days after transfer. Note giant cells. ×280.

FIG. 25.—Section of transplant of HC 305 in brain of a 6th-generation guinea pig. The animal was killed 71 days after transfer. Note palisading. ×160.

FIG. 26.—Section of transplant of HC 305 in brain of 2d-generation mouse, 40 days after transfer. ×280.

FIG. 27.—Section of transplant of HC 305 in brain of a 1st-generation mouse killed 88 days after transfer. Note palisading. ×125.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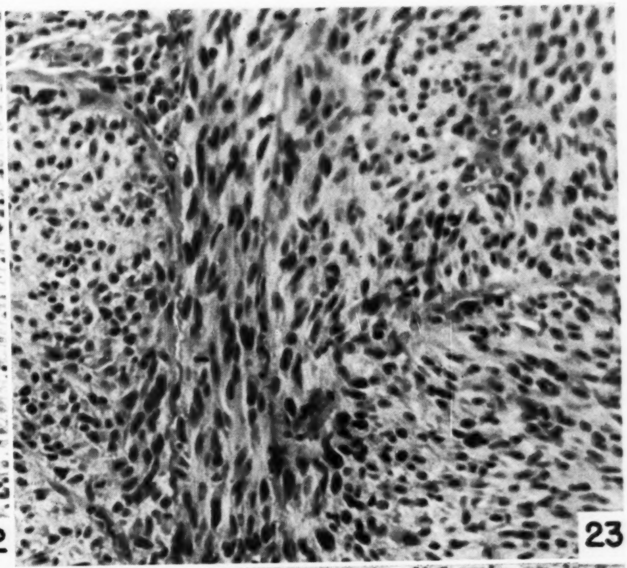
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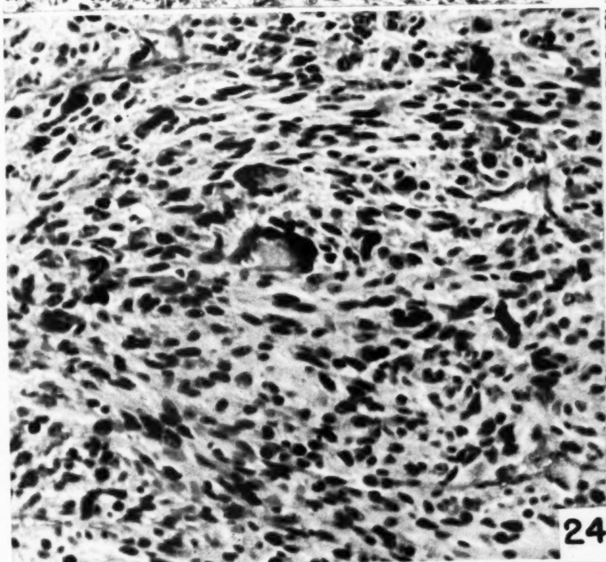
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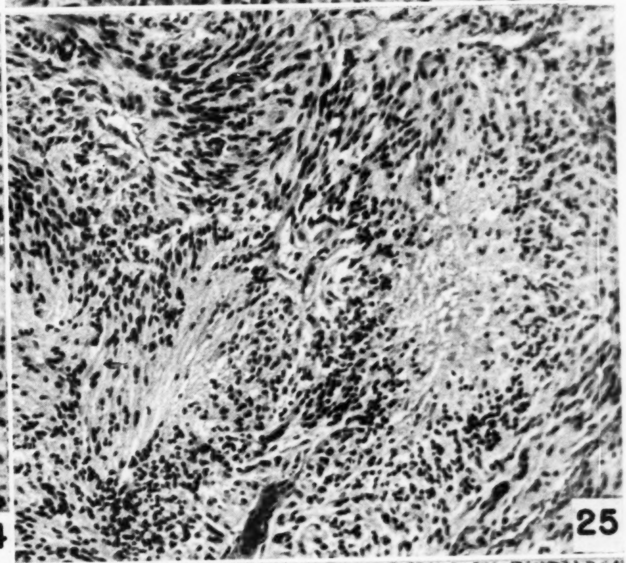
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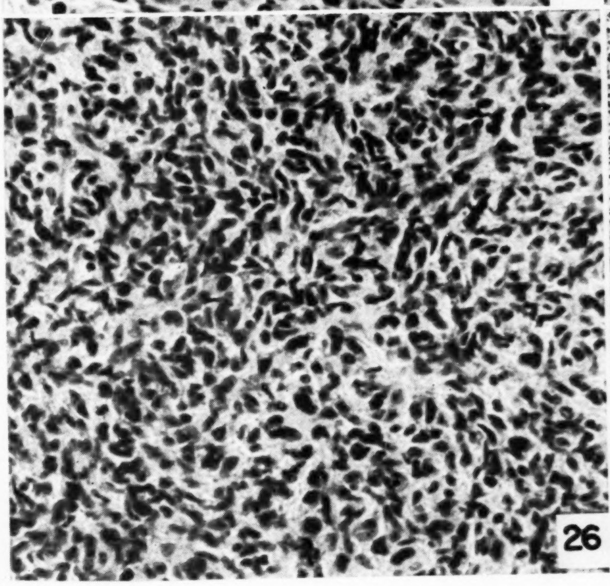
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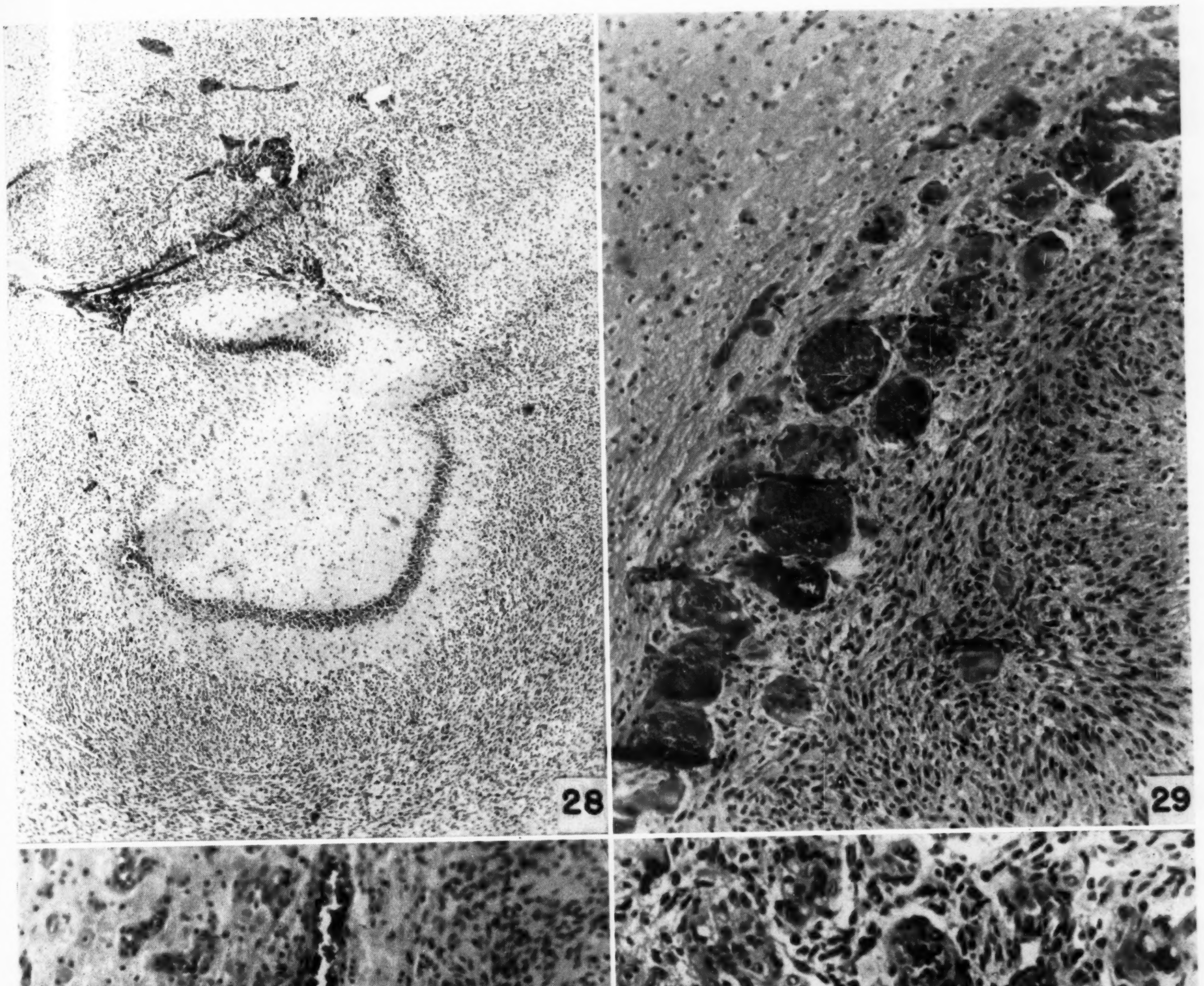
FIG. 28.—Section of transplant of HC 305 in brain of a 1st-generation mouse, killed 94 days after transfer. Note preservation of island of normal brain tissue in midst of tumor. ×50.

FIG. 29.—Section of transplant of HC 305 in brain of a 4th-generation guinea pig killed 91 days after transfer. Note abundant vascular channels at edge of tumor. ×130.

FIG. 30.—Section of transplant of HC 305 in brain of an 8th-generation guinea pig killed 54 days after transfer. Note endothelial proliferation in capillaries of normal brain at edge of tumor. ×150.

FIG. 31.—Section of transplant of HC 305 in brain of a 5th-generation guinea pig killed 78 days after transfer. Note capillary endothelial proliferation in tumor. ×280.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The Promoting Action of Croton Oil in Skin Tumorigenesis*

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It has been observed repeatedly in mice that skin tumors may be induced by a single painting of a carcinogen when followed by repeated painting of the noncarcinogen, croton oil (1, 3, 6, 7, 9). If croton oil is not applied, few or no tumors appear, unless the amount of carcinogen applied initially is excessive (3). It has been suggested that "latent tumor cells" or cells with "latent neoplastic potentialities" are produced following application of a carcinogen and that these altered cells may be stimulated by a noncarcinogen (i.e., wounding, turpentine, croton oil) with the resultant formation of visible tumors (1, 4, 5). The former stage has been referred to as initiation and the latter as promotion (1, 4). In those experiments where the promoting action of croton oil was established, repeated paintings were employed (1, 3, 6, 7, 9). Since initiation occurred following one painting with a carcinogen, the question arose whether one painting with croton oil would similarly provide a sufficient stimulus for promotion. If not, how many additional paintings were required? Was continuous painting with croton oil more effective than intermittent? Finally, did promotion consist of a series of irreversible changes which were additive or were they reversible? If the former, this should become evident among mice painted intermittently with croton oil. These and related questions have been investigated and form the basis for the present study.

MATERIALS AND METHODS

Strain DBA/2 JAX male mice 34–70 days of age were employed in these experiments. The animals were housed in metal cages in an air-conditioned room (temperature 76°–78° F.) and had access to a continual supply of Purina Laboratory Chow pellets and tap water.

The carcinogen 20-methylcholanthrene (m.p. 179°–180° C.), referred to hereafter as MCA, was prepared as a 0.5 per cent solution in U.S.P. grade olive oil. Croton oil[1] was applied as a 5 or 10 per cent solution in olive oil. Solutions of MCA and croton oil were painted on both ears of each mouse with a No. 3 camel-hair brush. The mice received a single application of the carcinogen, were segregated one to a cage for 2 days, and were reassembled and maintained ten to a cage for the duration of the experiment. Skin painting with the croton oil solution was begun approximately 2 weeks following application of the carcinogen. Some of the mice showed toxic symptoms as a result of repeated applications of croton oil. Under these circumstances, painting was withheld for a sufficient interval to permit recovery and was then renewed.

The mice were examined for skin tumors 2 months following application of the MCA and then weekly throughout the experiment. Tumors were not recorded unless they reached a minimum size of 1 mm. and persisted at least 2 weeks.

The experiment was divided into two stages, the same mice being used in both.

In Stage I, all the mice were painted once with the MCA solution, set aside for 14–20 days, and then divided into six groups according to treatment with croton oil as follows:

Group A.—Nine mice were painted once with a 10 per cent solution of croton oil.

Group B.—Thirty mice were painted once with a 5 per cent solution of croton oil.

Groups C–F.—Thirty mice each were painted 3 times weekly with a 5 per cent solution of croton oil. The mice received a total of five paintings in Group C, ten in Group D, twenty in Group E, and 30 in Group F.

At the termination of croton oil painting, the mice in each group except Group A were maintained an additional 20 weeks without further treatment. (In Group A, this period lasted 26 weeks, after which interval the nine survivors were sacrificed.) This marks the end of Stage I.

Immediately following the 20-week period of nontreatment described in Stage I, all surviving mice from Groups B–F were again painted on the ears with the 5 per cent solution of croton oil—this time twice weekly for 19–20 weeks. In contrast to the condition in Stage I, each mouse now received the same number of applications of croton oil regardless of group. The experiment was terminated 40 days following the last application of croton oil; the mice were sacrificed, and appropriate tissues excised and fixed for further study.

RESULTS

The incidence of tumors is presented at three stages during the experiment: (*a*) at the end of Stage I (Table 1); (*b*) during the course of Stage

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[1] Obtained from the Fisher Scientific Company, Eimer & Amend, New York, N.Y.

II at a time when the period of observation was the same and the total applications of croton oil similar in all groups (Table 2); and (*c*) at the end of Stage II (Table 3).

The results obtained following the first series of paintings with croton oil are summarized in Table 1. Although the nine mice in Group A were observed for a total of 202 days, no visible skin tumors were recorded. In Group B, two mice out of 28 bore such tumors within an observation period of 155 days. These appeared 88 and 131 days following the application of MCA but regressed within the next 30 days. In Group C, the mice were observed 163 days; none of the 29 survivors bore a tumor. Similarly, no tumors were induced among the mice in Group D which were observed 176 days. One mouse in 25 in Group E bore a papilloma 62 days after MCA was applied. This tumor persisted for the duration of the experiment. More tumors were induced in Group F than in the other five groups combined. Thus, six mice out of 29 were susceptible after an average latent period of 124 days. Three of these tumors persisted, while the other three regressed after 30, 45, and 128 days, respectively. In order to compare tumor incidence in Group F with that in the other groups which were observed for shorter periods, a record was made of the number of tumors observed in Group F 155 days after the start of the experiment. This result is listed as Group F1 in Table 1. Despite the reduction in time of observation from 230 to 155 days, more tumors were observed in Group F1 than in any of the remaining groups (A–E).

Table 2 is a summary of results for the mice in Groups B–E after an observation period of 230 days. This corresponds with the observation period for Group F, Table 1, which is repeated for convenience in Table 2. Under the conditions of the experiment, extension of the period of observation resulted in a renewal of painting with croton oil in Groups B–E. Thus, the mice in these groups were again exposed to croton oil 20 weeks following the last application described in Stage I. As a result, all the mice in Table 2 were exposed to approximately the same total number of applications of croton oil. Although tumor incidence increased slightly in most of the groups in Table 2 compared to that in the corresponding groups in Table 1, it still was appreciably lower than that observed in Group F.

Many more mice with tumors were observed

TABLE 1

EFFECT OF NUMBER OF APPLICATIONS OF CROTON OIL ON CO-CARCINOGENESIS

Strain DBA male mice were painted once on the skin with a 0.5 per cent solution of MCA. Approximately 2 weeks thereafter, painting with 5 per cent croton oil was begun.

Groups	Survivors at time of first tumor	Total applications croton oil	Last application croton oil* (days)	Mice with tumors	Tumor incidence (Per cent)	Total tumors	Av. latent period* (days)	Av. observation period* (days)
A	9	1†	15	0	0	0		202
B	28	1	15	2	7	2	109	155
C	29	5	24	0	0	0		163
D	28	10	36	0	0	0		176
E	25	20	60	1	4	1	62	197
F	29	30	85	6	21	6	124	230
F1	29	30	85	5	17	5	104	155

* Measured from initial painting with MCA.
† Painted with 10 per cent solution of croton oil.

TABLE 2

INFLUENCE OF CONTINUED VERSUS INTERRUPTED TREATMENT WITH CROTON OIL ON CO-CARCINOGENESIS

All mice were painted once with a 0.5 per cent solution of MCA prior to treatment with croton oil. A period of 20 weeks of nontreatment followed. Painting with croton oil was then resumed and was continued for the remainder of the observation period.

Groups	Survivors at time of first tumor	Initial paintings with croton oil	Total applications croton oil (Av.)	Mice with tumors	Tumor incidence (Per cent)	Total tumors	Av. latent period* (days)	Av. observation period* (days)
B	28	1	23	3	11	3	149	230
C	29	5	25	2	7	2	202	230
D	28	10	26	1	4	1	223	230
E	25	20	30	1	4	1	62	230
F†	29		30	6	21	6	124	230

* Measured from initial painting with MCA.
† Continued treatment group from Table 1 repeated for convenience.

following the second period of treatment with croton oil as described under Stage II above. The total number of applications of croton oil varied from 39 in Group B to 64 in Group F (Table 3). Tumor incidence now ranged from 43 to 56 per cent, while the average number of tumors per mouse increased slightly (Table 3). Latent period measured from the start of renewed treatment with croton oil did not vary much among Groups B–F (102–118 days). This interval was comparable to the latent period observed for Group F in Table 1.

Although one of the tumors induced in Stage II developed into a squamous-cell carcinoma, the others did not progress beyond the benign, papillomatous stage. Before a growth was recorded as a tumor, it had to measure at least 1 mm. in diameter and, in addition, persist a minimum of 2 weeks. Several days before the end of the experiment, ten growths were observed among mice not previously susceptible, as well as ten in others already bearing tumors. Since these new growths, some of which exceeded 1 mm. in size, had not been observed 14 days at time of autopsy, they were not recorded in Table 3.

DISCUSSION AND CONCLUSIONS

When croton oil was applied once to an area of skin previously painted with MCA, few or no visible tumors were observed (Groups A, B, Table 1). In a recent study of skin tumorigenesis, Salaman (8) painted mice once with 9,10-dimethyl-1,2-benzanthracene and then once with croton oil, and also reported a lack of skin tumors. It is evident from these experiments that a repeated application of croton oil is needed for effective co-carcinogenic action. Thus, a promoting effect was observed only after mice had received 30 paintings (Group F, Table 1). These results demonstrate that promotion, unlike initiation, is a gradual process.

It will be observed that the period of observation was longer in Group F than in the other groups (Table 1). To eliminate the possibility that this may have contributed to the higher tumor incidence observed in Group F, a count was made of the tumors which had appeared within the first 155 days of the experiment. This interval corresponded to the shortest observation period in Table 1. The result, entered as Group F1, showed that tumor incidence was not altered significantly, for it decreased only from 21 to 17 per cent. This indicates that the high tumor yield in Group F (Table 1) probably was due to the longer period of painting with croton oil.

After a lapse of 20 weeks, the mice in Groups B–E were again painted with croton oil. This afforded an opportunity to compare the influence of continued as against interrupted treatment with croton oil on carcinogenesis. The "continued" group, represented by Group F, was composed of mice which had been painted repeatedly with croton oil and in which effective promoting action had been observed. The "interrupted" series was represented by Groups B–E. As may be seen in Table 2, the average period of observation and the total applications of croton oil in all the groups were the same, or nearly so. Despite this similarity in treatment, however, the number of mice with tumors in Group F exceeded that in any of the other groups and almost equaled the tumor yield for all the other groups combined (Table 2). In Groups E and F, the total number of paintings with croton oil was identical. However, tumor incidence in these two groups was 4 and 21 per cent, respectively. Thus, it may be concluded that a continued series of paintings with croton oil is more effective in co-carcinogenesis than an interrupted one.

The mice in Groups B–F which initially had received one to 30 paintings with croton oil, respectively, were again painted with this promoter. Now, however, the mice received approximately the same number of paintings, and these were

TABLE 3

CO-CARCINOGENESIS IN STRAIN DBA MALE MICE FOLLOWING RENEWAL OF SKIN PAINTING WITH CROTON OIL

All mice initially were painted once with MCA and then 1 to 30 times with croton oil.
Following a 20-week period of nontreatment, painting with croton oil was resumed.

Groups	Total mice*	Renewed paintings with croton oil (Av.)	Total applications croton oil (Av.)	Mice with tumors	Tumor incidence (Per cent)	Total tumors	Av. tumors per tumor bearer	Av. latent period† (days)	Av. observation period† (days)	Total observation period from MCA (days)
B	28	38	39	12	43	21	2	118	173	328
C	27	40	45	15	56	17	1	102	172	335
D	26	39	49	14	54	21	2	109	173	349
E	23	39	59	12	52	15	1	111	178	375
F	23	34	64	10	43	14	1	106	172	402

* Survivors at time of first tumor following renewed treatment with croton oil.
† Measured from start of renewed painting with croton oil.

administered over a similar period of time (Table 3). At autopsy, the incidence of tumors in Groups B–F varied from 43 to 56 per cent. If the promoting influence resulting from the initial series of skin paintings with croton oil had produced lasting or permanent alterations in the direction of neoplasia among the latent tumor cells or cells with latent neoplastic potentialities already present, one would have anticipated a rise in tumor incidence in going from Group B to F (Table 3). Thus, a 21 per cent tumor incidence already had been observed in Group F before painting with croton oil was resumed (Table 1), and it is likely that other tumors were also present but had not yet progressed to the point of visibility. Despite this advantage, tumor incidence in Group F following the second period of painting was as low as or lower than that in any of the other groups (Table 3), indicating that the alterations produced in the skin by the first series of paintings with croton oil probably had regressed during the 20-week interim period of nontreatment. In contrast to this observation on the reversibility of the promoting influence is the observation of Berenblum and Shubik (2) that one application of a carcinogen initiates a lasting or irreversible effect on the skin, which may be demonstrated even after a lapse of 43 weeks.

Another indication that the initial treatment with croton oil had failed to influence subsequent co-carcinogenic action may be seen from a comparison of latent periods for Group F following the first and second periods of treatment. Thus, counting from the initial application of MCA, the latent period was 124 days, or 104 days when measured from the first application of croton oil (Table 1), while a latent period of 106 days was observed following the renewed series of paintings with croton oil (Table 3). In Salaman's experiments (8), some of the mice were painted continuously with croton oil, while others received croton oil during two different intervals, a rest period intervening. Since it was observed that the latent period did not vary significantly in the "continuous" and "intermittent" groups, the author concluded that the skin in the latter group responded as though it had never been exposed to croton oil prior to the start of the second series of paintings. The results in the present experiment serve to reinforce this conclusion.

The mice in Group F were given three paintings per week of croton oil during Stage I, while during Stage II they were painted only twice per week. These mice received approximately the same number of continued paintings in both Stages—30 versus 34. Despite a shorter observation period, tumor incidence for Group F in Stage II (Table 3) was more than twice that in Stage I (Table 1). It would appear, therefore, that co-carcinogenic action is more effective when croton oil is applied twice weekly than when applied 3 times per week. The greater amount of skin damage which is apparent following the latter mode of treatment may be directly related to the observed decrease in tumor incidence.

SUMMARY

Strain DBA mice were painted once on both ears with methylcholanthrene. Approximately 2 weeks thereafter, croton oil was applied to the same site. Although few or no tumors were observed following 1, 5, 10, or 20 paintings with croton oil, a definite co-carcinogenic effect was obtained when the number of applications was increased to 30.

When croton oil was painted continuously during an extended interval and, following a rest period, this was repeated, it was observed that the first series of paintings had little influence on the second in promoting the development of visible tumors.

Croton oil was more effective as a promoter when applied continuously rather than intermittently and when applied 2 times as against 3 times per week.

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Effect of Various Compounds on the Ehrlich Ascites Carcinoma*†

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Since the first description of the Ehrlich ascites tumor in 1932 by Loewenthal and Jahn (24), the ascites tumor has been used comparatively infrequently by investigators for chemotherapeutic studies. Collier has noted the high resistance of the tumor cells *in vitro* to chemical agents (6) and to changes of hydrogen ion concentration (5). Lettré (21) has shown that trypaflavine has an inhibitory effect on the growth of the ascites tumor, and his observation was confirmed by Klein (13). The inhibition was found to be most definite when trypaflavine treatment was started soon after the inoculation, while treatment of the established ascites tumor was less effective. Lettré (20, 22) and Klein (14) have found that intraperitoneal injections of colchicine had an inhibitory effect on the development of ascites tumor and markedly prolonged the survival time of treated mice. On the other hand, Schairer (33) could not obtain a successful inhibition of the Ehrlich ascites tumor with colchicine unless the administered doses reached the toxic level. Stilbamidine (18) has no effect on the Ehrlich ascites tumor. Recently, Klein (14) has reported that intraperitoneal injections of ACTH and HN2 had no effect on the growth rate of the Ehrlich ascites tumor. Aminopterin and A-methopterin had an inhibitory effect, but only in highly toxic doses. Klein also found that Shear's bacterial polysaccharide, when injected intraperitoneally into mice bearing Sarcoma 37 (solid and ascitic forms), produced a marked hemorrhage and extensive necrosis in the solid tumors. On the other hand, the ascitic fluids showed only an increased inflammatory reaction, without any change in the morphology of the tumor cells. Besides the above-mentioned compounds, the following chemicals have also been tested against the growth of Ehrlich ascites tumor: D-riboflavin, ethyl bromoacetate, fluorotyrosine, urethan, porphyrine, adenosine-5-phosphate, adenosine-3-phosphate, adenosine triphosphate, 3,4-benzpyrene, 9,10-dimethyl-1,2-benzanthracene, cysteine, and heavy metal salts (1, 2, 3, 7, 9, 10, 15, 16, 19, 23, 27, 34). Some of the compounds showed activity against this tumor. In Japan, investigators have employed the Yoshida ascites sarcoma for chemotherapeutic studies (12, 17, 28, 29, 30, 31, 32, 46, 48). Thus, Yoshida (47) found that intraperitoneal injections of colchicine, papaverine, potassium arsenite, neo-salvarsan, and chloralhydrate showed destructive action upon the tumor cells and increased the survival time of rats twice over that of the untreated animals. Miyaji *et al.* (25) and Yoshida *et al.* (50) have noted that intraperitoneal injections of a nitrogen mustard and of aminopterin had a destructive action on ascites sarcoma cells and prolonged the survival time of treated rats. Ishidate *et al.* (11, 49) investigated the cytotoxic activity of N-oxide derivatives of nitrogen mustards against the Yoshida ascites sarcoma in rats. They found methyl-bis(β-chloroethyl)amine N-oxide to be the most effective tumor growth-inhibitor among the oxides of nitrogen mustards tested.

Previous investigations with a spectrum of tumors (fifteen mouse and five rat tumors) demonstrated that several compounds had a definite inhibitory or destructive action on certain types of tumors (26, 37, 39, 40, 43, 44). In view of anticipated interest in ascites tumors, it was believed that tests against the Ehrlich ascites carcinoma of compounds of demonstrated or suggested activity against other tumors would be useful as a base-line for future studies with this tumor.

MATERIALS AND METHODS

The Ehrlich mouse ascites tumor[1] was chosen for the present study. This ascites tumor was obtained from the Ehrlich mouse carcinoma, which

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[1] The Ehrlich ascites tumor was kindly supplied to us by Dr. George Klein, Karolinska Institutet, Stockholm, on October 1, 1950, to whom we wish to express our sincere thanks.

originated as a tumor of the mammary gland. Intraperitoneal injection of the tumor emulsion produces ascites (24). Solid tumors are obtained by subcutaneous injection of fresh ascitic fluid containing cancer cells, and ascites tumors are obtained by intraperitoneal injection into mice.

Upon intraperitoneal injection of fresh ascitic fluid containing about 1 million cancer cells, mice regularly develop large amounts of milky ascites (5–20 cc.) in 7–14 days (see Fig. 4) and die in 2–6 weeks. The tumor generally has 100 per cent takes and regresses in about 5 per cent.

Figure 1 shows a section of the solid form of the Ehrlich ascites tumor, composed of solid sheets of rounded cells. Figure 2 shows a smear preparation of ascitic fluid of the Ehrlich ascites tumor. The large cells are cancer cells, and small cells are normal cells (leukocytes, histiocytes, mesothelial cells).[2] Many mitotic figures are seen in the section. Figure 3 shows an electron micrograph of a fresh ascites tumor cell. It shows clearly the cytoplasm, nucleus, chromatin, mitochondria, and processes or so-called spikes that indicate great activity.

The milky fluid contains about 5 to 100 million cancer cells/cc—usually about 25 million cancer cells, and about 5–10 per cent of normal cells. Cancer cells in ascitic form are much larger than those in solid form.

Hemorrhagic exudates were occasionally observed in 12–14-day-old ascites tumors. Inoculation of the ascitic fluid containing a fairly large number of erythrocytes had no inhibitory effect on the normal course of ascites formation. The frequency of appearance of hemorrhagic ascites increased with time, so that at 21 days as many as 50 per cent of animals showed hemorrhagic ascites. At this time the fluid had a tendency to clot.

Intraperitoneal injections of 0.1–0.2 cc. of fresh ascitic fluid caused formation of solid tumors at the peritoneal wall at the site of the needle insertion in as many as 80 per cent of the cases. However, by using a fine needle with special care this number was greatly reduced.

Mice regularly developed solid tumors after subcutaneous injection of 0.1–0.2 cc. of fresh ascitic fluid containing 1 to 2 million cancer cells. The tumors grew rapidly and, 14 days after injection, reached a size of approximately 19 × 12 × 7 mm. Many tumors penetrated into the abdominal wall. There was no formation of tumor ascites. However, in one of the 30 mice about 3 cc. of hemorrhagic exudate were found. Visceral metastases did not occur.

A series of intravenous inoculations of Ehrlich ascites tumor was performed. Fresh ascitic fluid (0.1 cc.), containing a known number of cancer cells, was injected into each of 30 young mice through a vein in the tail. Of ten mice that received 3 million cancer cells, two died from shock soon after the injection. No deaths from shock occurred among the remaining twenty animals, which had received either 1 or 2 million cancer cells. The results obtained from these experiments are briefly summarized. Of the 27 mice living more than 19 days, seventeen animals, or 63 per cent, developed many tumor nodules in the lung; thirteen animals, or 48 per cent, developed solitary tumors in the heart; twenty animals, or 74 per cent, showed solid tumors in the tail, at the site of the injection. Other mice had large solid tumors at the base of the tail. Many of the mice which had carcinomas in the lungs also showed fluid in the pleural cavity, whereas tumor ascites was not present in the abdominal cavity. No growths were observed in the liver, spleen, and kidney.

To study the influence of the genetic constitution of the host upon the development of tumor ascites, 0.1 cc. of ascitic fluid, containing 1 to 2 million cancer cells, was injected intraperitoneally into each of 200 mice of different strains. The strains were: Rockland, CFW, Banks and AKm albino mice; C57BL; C3H, and DBA (8). The results showed that the Ehrlich ascites tumor grows equally well in the abdominal cavity of each of these strains of mice.

Our previous study with certain transplantable tumors indicated that the age of the host had a definite influence upon the number of "takes," the growth rate, and the regression of tumor transplants (38). The present work extends this study to the transplantable Ehrlich ascites tumor; 0.05 cc. of ascitic fluid containing 0.05 to 1 million cancer cells was injected intraperitoneally into suckling mice (5–12 days old). Tumor ascites developed in all the 44 animals and killed them within 2 weeks. Lung metastases were seen in most cases. Male and female mice 4–6 weeks old were found to be equally susceptible to the tumor, and animals of this age group offer most satisfactory conditions for experiments of this kind. Tumor ascites did not develop well in old mice (about 6 months old) nor in sick mice.

Intraperitoneal injection of 0.1 cc. of the ascitic fluid, containing 1 to 4 million cancer cells, into young healthy rats (about 30 days old) failed to produce tumor ascites. About 50 per cent of similarly treated suckling rats (0.5 to 6 days old) developed ascites, and of these about one-half continued to accumulate ascitic fluid until death.

Unlike solid tumors the Ehrlich ascites tumor deteriorates rapidly on standing at a temperature of 3°–4° C. The bottles containing tumor ascites were allowed to remain for definite periods of time in the refrigerator at 3°–4° C. At the end of these periods, samples of the tumor ascites (0.1 cc. containing 1 to 2 million cancer cells) were injected intraperitoneally into young mice. The results showed that the growth capacity of the tumor ascites was almost completely lost after 5 days: 90 per cent of the animals failed to develop ascites. After 1–3 days of standing, 20–50 per cent growth occurred (a total of 100 animals was used), and the appearance of ascites was greatly delayed.

The average rectal temperature of normal mice was 100° F., while mice with 14-day-old ascites tumors had an average rectal temperature of 101° F., and in the terminal stages (3-week-old ascites) the temperature was 98° F. Thus, peritonitis would appear to have been absent.

There are several ways of determining the effectiveness of a compound against the ascitic form of tumors. One is to examine the ascites tumor cells cytologically after injection of a compound; another is to measure increase in survival time resulting from the therapy; a third is to measure the amount of ascites formed after treatment. We chose the latter method.

[2] The author wishes to acknowledge his indebtedness to the late Dr. J. Scapier and Mrs. G. R. Durfee for cytological study of the Ehrlich ascites tumor.

Preliminary experiments were made to determine the relation between the number of inoculated cancer cells and the rate of accumulation of ascitic fluid, the spontaneous regression of ascites tumors, and the length of life of the tumor-bearing mice. The results obtained from these studies are summarized in Table 1. Each inoculum was tested in a group consisting of ten mice. Therefore, the listing of 100 mice in column 1 indicates ten separate experiments. The mice were male albinos (Rockland Farms), 5–6 weeks of age (18–22 gm.).

It is clear that intraperitoneal injections of 0.1–0.2 cc. of 7- to 14-day-old ascites containing 5 to 10 million cancer cells per mouse resulted in development of ascites tumors in 100 per cent of the mice with no spontaneous regression. All animals showed solid tumors on the peritoneal wall at the site of injection. Many animals died in 1–2 weeks, but some lived for 4 weeks. With inoculation of 1 million cancer cells, ascites tumors developed generally in 100 per cent of the mice within 2 weeks with about 5 per cent regressions. It is interesting to note that the number of solid tumors formed at the peritoneal wall at the site of inoculation of ascitic fluid sharply dropped—from 100 per cent to 38 per cent. The average survival time of animals, however, was essentially the same as that following inoculation of 5 million cancer cells.

Ascites tumor formation was greatly decreased with decreasing number of cells inoculated. This was evident with 50,000 cells or less. Intraperitoneal injection of 50 cancer cells failed to develop ascites tumor or solid tumor at the site of injection.

The survival time of mice having ascites following inoculation of 50,000 cells or less was definitely increased. This was partly due to the delayed appearance of ascites or solid tumors. In some animals only solid tumors were produced at the peritoneal wall at the site of injection.

With solid tumors, it is possible to measure tumor volume or weight directly at various intervals of time after subcutaneous transplantation of tumor fragments. In ascites tumors, weight measurements of animals bearing the ascites may be the best way of following tumor development. Lettré (20, 22) has pointed out that weight increase has a direct relationship to the ascites formation. This has been questioned by Klein (14). We made the following experiments to find the relation between weight increase and abdominal distention.

When fresh ascitic fluid (0.1 cc. containing about 1 million cancer cells) was injected into the peritoneal cavity of mice, the cells proliferated at the surfaces of visceral and parietal peritoneum. During the first 3 days, neither the cell nests nor ascites were observable grossly. However, saline washings of the peritoneal cavity showed that the number of cancer cells had increased several times compared to the original number. At the end of the 4th day one could see at autopsy a small amount of ascitic fluid—about 1.0 cc., and swelling of intestine, omentum, and mesentery was observed. At the end of the 7th day mice showed a slight to marked abdominal enlargement. These animals had from 2 to 18 cc. of milky white fluid in their peritoneal cavity. However, at this time some animals did not have any ascitic fluid. At this time also one could observe a small tumor nodule in the peritoneal wall at the site of ascites injection and foci of tumor cells on the peritoneal surfaces of intestine and around the pancreas. These solid tumors grew rapidly, and, 14 days after inoculation of ascites, they reached a size of approximately 10 × 7 × 5 mm. The solid tumors continued to grow rapidly, and the animals finally succumbed even without ascites.

On the 14th day, almost all animals showed a moderate to marked abdominal distention. Such animals had from 5 to 25 cc. of ascitic fluid. At this time, there were large and small solid tumors in the abdominal wall, peritoneal surfaces of the liver, spleen, diaphragm, intestine, retroperitoneal space, pelvic cavity, and around the pancreas; cancer in the mesentery, omentum, and lymph nodes; and metastases in the lungs. No metastases were found in the heart, kidney, adrenal, liver, and spleen. On the 21st day many animals showed marked abdominal distention and carried from 20 to 50 cc. of ascitic fluid. These fluids were much less turbid and contained many fewer cancer cells per cc. than those of 7- to 14-day-old ascites.

TABLE 1

EFFECT OF NUMBER OF TUMOR CELLS INOCULATED ON PERCENTAGE OF TUMOR GROWTH AND SURVIVAL TIME OF MICE

No. mice	No. cells	Per cent of tumor growth: Ascitic form	Per cent of tumor growth: Solid form*	Survival time of tumor-bearing animals (weeks)
20	10×10^6	100	100	1–3
20	5×10^6	100	100	2–4
50	3×10^6	96	80	2–5
100	2×10^6	95	60	2–6
100	1×10^6	95	38	2–6
50	5×10^5	82	38	2–6
30	3×10^5	60	30	3–5
20	2×10^5	80	40	3–6
20	1×10^5	60	10	3–4
30	5×10^4	13	33	3–4
20	2×10^4	20	30	3–4
30	1×10^4	46	30	3–6
20	5×10^3	40	40	5–6
20	5×10^2	20	20	4–6
20	1×10^2	20	20	7–8
20	5×10^1	0	0	

* Solid tumors at the peritoneal wall at the site of injection.

A typical example of growth curves of mice following the inoculation of fresh ascitic fluid containing about 1 million cancer cells is shown in Chart 1. In some experiments, however, we obtained entirely different weight curves owing to individual variations in tumor susceptibility. Some animals gained little weight, others none at all, which indicates no ascites formation.

The above experiments showed that the increase of abdominal distention correlates fairly well with the weight increase or volume of the fluid. Therefore, the volume increase of ascites was graded according to the following scheme: − indicates no abdominal enlargement (no gross ascites); ± indicates slight ascites (1 to 5 cc. ascites); + indicates moderate abdominal enlargement (5 to 15 cc. ascites); and ++ indicates marked abdominal enlargement (15 to 50 cc. ascites).

Chemotherapy test.—We adopted the following procedure for chemotherapeutic studies with the Ehrlich ascites tumor as a test object. A donor mouse was selected among mice having rapidly accumulating ascites (moderate to marked abdominal distention) at 7–14 days after inoculation of cancer cells. About 5–10 cc. of milky ascitic

fluid were withdrawn with a syringe having a 20-gauge needle. Then the cells were counted with a hemocytometer, and a proper dilution was made with physiological salt solution at pH 7.0. Intraperitoneal injection of 0.1 cc. of the fluid containing about 1 million cancer cells into young healthy

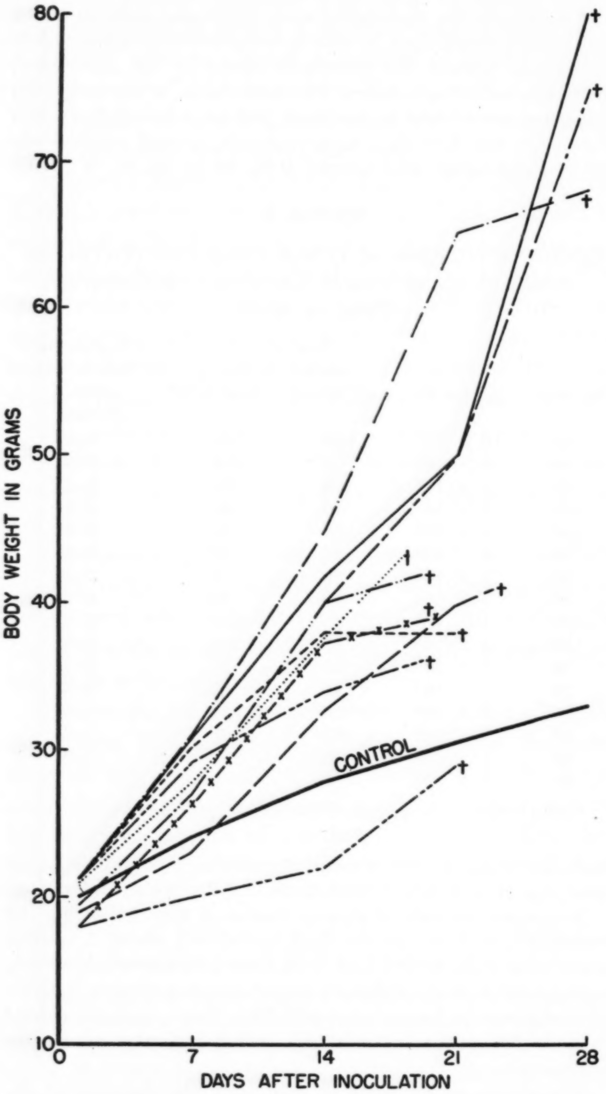

Chart 1.—Weight curves of mice after injection of 1,000,000 Ehrlich ascites tumor cells. The heavy continuous line (control) indicates the average growth curve of 30 normal young male mice. Each of the other lines indicates the growth of one ascites-injected young male mouse. Death is indicated by a cross (†).

mice was made in the inguinal region with a $\frac{1}{4}$-cc. syringe having a 23-gauge needle. Aseptic precautions were maintained during the procedure. The animals were maintained on a standard pellet diet (Purina Laboratory Chow) and water.

In every set of experiments, ascites tumor-bearing animals were divided into two groups: one to be treated with the compounds and the other to be used as controls. The progress of the ascites tumors in the animals was recorded by weight measurements of animals at weekly intervals. In certain experiments, the influence of compounds was evaluated by measuring the amounts of ascitic fluids at the end of 2 weeks after the first injection of compounds.

The degree of inhibition of ascites tumor growth was graded according to the following scheme:

Marked inhibition (++) indicates no abdominal distention, or normal gains in body weight of animal—about 0.6 gm/day.[3]

Moderate inhibition (+) indicates slight abdominal distention, or 2 times the normal gain in body weight.

Slight inhibition (±) indicates moderate abdominal distention.

No effect (−) indicates marked abdominal distention, or 4 times the normal gain in body weight or more.

We are aware that this is not a particularly satisfactory way to measure the quantity of tumor ascites in the treated and nontreated animals. The final results are based upon (*a*) the estimations of abdominal distention at the end of the 2d week compared to those of controls and (*b*) the survival time of animals during the experimental period of 6 weeks.

In the case of solid Ehrlich carcinoma, carefully selected tumors were cut into 1.5-mm. cubes and implanted subcutaneously into the axillary region of healthy young mice by the usual trocar method. The course of development of the tumors in the animals was recorded graphically by measuring them in two diameters with calipers every week.

The solid form of the Ehrlich carcinoma grows rapidly, and, 14 days after transplantation, the tumors reach a size of approximately 18 × 17 × 11 mm. The tumor transplants take in 100 per cent of cases without regressions.

The retarding effect upon solid tumor growth was graded according to the following scheme:

- ++ indicates marked inhibition; the tumors failed to grow or grew to approximately one-fourth the average diameter of the controls.
- \+ indicates moderate inhibition; the tumors averaged from one-fourth to one-half the diameter of the controls.
- ± indicates slight inhibition; the tumors grew to from one-half to three-fourths the diameter of the controls.
- − indicates no effect; the tumors grew to three-fourths or more the diameter of the controls.

The first intraperitoneal or subcutaneous injection of compounds was given 1 day after inoculation of tumor fragments or injection of ascitic fluid, and injections were continued for 7 days.[4]

[3] In some animals the daily weight increase may be much less due to toxic action of compounds. Survival of more than 50 per cent of treated animals beyond 3 weeks was considered as a marked effect.

[4] We wish to acknowledge the co-operation of the following laboratories for supplying the compounds used in the study: Abbott Laboratories; Armour & Company; Ayerst, McKenna & Harrison, Ltd.; Dr. Aaron Bendich, Sloan-Kettering Inst.; Calco Chemical Div., American Cyanamid Co.; Dr. Liebe Cavalieri, Sloan-Kettering Inst.; Chemical Specialties, Inc.; University of Chicago Toxicity Lab.; Ciba Pharmaceutical

RESULTS AND DISCUSSION

As a preliminary study, we tested compounds of unusual theoretical interest, in addition to those which showed activity against the other experimental tumors (26, 37, 39, 40, 41, 43, 44, 45). The results obtained with various compounds are presented in Table 2. This study included the use of 730 mice in the treatment group and 480 mice in the control group.

In the control group of 300 mice inoculated with about 1 million cancer cells in the abdomen, 296 animals survived 14 days or died earlier with moderate to marked abdominal distention. Of these 296 animals, 41, or 14 per cent, showed no abdominal distention (no gross ascites); 39 animals, or 13 per cent, showed slight abdominal distention; 72 animals, or 24 per cent, showed moderate abdominal distention; and 144 animals, or 49 per cent, showed marked abdominal distention (15 to 50 cc. ascites).

In this series of experiments there were 180 control mice implanted with the solid form of the Ehrlich carcinoma. The subcutaneous tumor transplants took in 100 per cent of cases, and only 1 per cent of them regressed. The tumors grew rapidly, and 14 days after transplantation they reached a size of approximately 14 × 12 × 9 mm.

Daily nontoxic doses of 0.5 mg/kg of colchicine, which is known as a good mitotic poison, had a slight inhibitory effect on Ehrlich ascites tumor when injected intraperitoneally. Daily intraperitoneal injections of 1.0 mg/kg of colchicine had a marked inhibitory effect on ascites, but many animals died. The compound had no inhibitory effect on the growth of solid Ehrlich mouse carcinoma.

Daily subcutaneous or intraperitoneal injections of folic acid (pteroyl glutamic acid) and antifolic acids aminopterin (4-amino-pteroyl glutamic acid) and A-methopterin (4-amino-N^{10}-methyl pteroyl glutamic acid) had no inhibitory effect on Ehrlich mouse carcinoma, in both solid form and ascitic form.

It is apparent from the results that daily subcutaneous or intraperitoneal injections of 0.5–1.0 mg/kg of NH2 (methylbis[2-chloroethyl]amine), caused a marked inhibitory effect on the development of ascites tumors in mice. More than 70 per cent of the treated animals failed to develop ascites, and the HN2 significantly prolonged the survival time of the animals beyond that of the controls. However, it had only a slight inhibitory effect on the growth of solid tumors.

HN2 has a definite damaging effect on the viability of Ehrlich ascites tumor at the level of 1.0 mg/kg/day. When mice bearing 7-day-old ascites tumors (showing slight to moderate abdominal distention) were given intraperitoneal injections of daily doses of 1.0 mg/kg of HN2, tumor growth stopped in about 2 days. Then about 50 per cent of 80 ascites regressed completely in 1–2 weeks (Fig. 4). Ascitic fluids tapped from mice 7 days after the first intraperitoneal injection, and then injected into normal mice, failed to produce ascites. Histological examination of abdominal fluids at the end of 7-day treatments revealed very few cancer cells, and they were all degenerated (Fig. 5). The small cells which are present in the fluids are normal cells, such as leukocytes, histiocytes, and mesothelial cells. Microscopic examination of abdominal fluids of untreated controls at the end of 14 days showed an abundance of active cancer cells and a small number of normal cells (see Fig. 2).

TABLE 2

EFFECT OF VARIOUS COMPOUNDS ON EHRLICH MOUSE CARCINOMA

Compound	Dose mg/kg	Solid form* Subcutaneous	Solid form* Intraperitoneal	Ascitic form* Subcutaneous	Ascitic form* Intraperitoneal
Colchicine	0.5	−	−	−	±
Folic acid	50	−	−	−	−
Aminopterin	0.25	−	−	−	−
A-methopterin	1.0	−	−	−	−
HN2	0.5–1.0	±	±	++	++
3-Bis(2-chloroethyl)aminomethyl-4-methoxymethyl-5-hydroxy-6-methylpyridine	5.0	±	±	±	++
Triethylene melamine	0.5	±	±	±	++
2,6-Diaminopurine	100	−	−	−	−
8-Azaguanine	75	−	−	−	±
Cortisone	37.5	±	−	++	±
Bacterial polysaccharide	5.0	−	−	−	++

* − = No effect.
± = Slight inhibition.
\+ = Moderate inhibition.
++ = Marked inhibition.

The daily subcutaneous or intraperitoneal injections of 5 mg/kg of a methoxy pyridoxine mustard (3-bis[2-chloroethyl]aminomethyl-4-methoxymethyl-5-hydroxy-6-methylpyridine), had a slight inhibitory effect on solid tumors, but there was a marked inhibitory effect on ascites when injected intraperitoneally.

Products, Inc.; Dr. Karl Folkers, Merck & Co.; B. F. Goodrich Co.; Dr. George H. Hitchings, Wellcome Research Laboratories; Hoffman-La Roche, Inc.; Lederle Laboratories Div., American Cyanamid Co.; Eli Lilly & Co.; Merck & Co.; Nat. Aniline Division, Allied Chemical & Dye Corp.; National Research Council; Parke, Davis & Co.; S. B. Penick & Co.; Dr. Karl Pfister, Merck & Co.; Chas. Pfizer & Co.; Roche-Organon; Rohm & Haas Co.; Schering Corp.; Dr. Murray J. Shear, Nat. Cancer Inst.; Smith, Kline & French Laboratories; Sterling-Winthrop Research Inst.; Upjohn Co.; and Dr. D. Wayne Woolley, Rockefeller Inst.

Repeated subcutaneous or intraperitoneal injections of 2,6-diaminopurine and 8-azaguanine had no inhibitory effect on Ehrlich mouse carcinoma, solid and ascitic forms.

Subcutaneous injections of 37.5 mg/kg of cortisone had a slight inhibitory effect on solid tumors and a marked inhibitory effect on tumor ascites. About 50 per cent of animals treated with cortisone failed to develop ascites during 4 weeks' observation. Many animals remained in good health. It is interesting to note that repeated intraperitoneal injections of 37.5 mg/kg of cortisone had only a slight inhibitory effect on ascites; at a higher toxic daily dose of 125 mg/kg it had a marked inhibitory effect, but many animals died.

We have treated a number of mice (40 animals) bearing well established 1-week-old ascites tumors with cortisone. Seven treatments of daily doses of 37.5 mg/kg of cortisone resulted in complete regression of ascites in about 50 per cent of cases.

TABLE 3

EFFECT OF UNDERFEEDING UPON THE GROWTH OF EHRLICH MOUSE CARCINOMA*

(Solid Form and Ascitic Form)

1.0 gm. food/day/mouse	= slight inhibition
1.5 gm. food/day/mouse	= slight inhibition
2.0 gm. food/day/mouse	= no effect
3.0 gm. food/day/mouse	= no effect
4.0 gm. food/day/mouse	= no effect
5.0 gm. food/day/mouse	= no effect
ad libitum	= no effect

* Intake of Purina Laboratory Chow was determined per cage of ten mice by daily loss of weight of feed in a special cup; tumor inhibition was graded from abdominal distention, weight gain, and survival, as detailed on p. 434.

In the course of the investigation, a polysaccharide from *Serratia marcescens* was used to treat a number of mice bearing the solid and the ascitic form of Ehrlich carcinoma. Shear has reported (35, 36) that bacterial polysaccharide, when injected into animals bearing tumors, may induce severe hemorrhage in tumors and may frequently result in the complete regression of the tumors. At our laboratory, we found that a single intraperitoneal injection of Shear's bacterial polysaccharide (Batch P-35, obtained by the courtesy of Dr. M. J. Shear), 5 μg/gm of mouse, induced hemorrhage (slight to severe) in 7-day-old Sarcoma 180 in 50 per cent of the mice, and 10 μg/gm induced good hemorrhage in almost 100 per cent. About 30 per cent of the tumors with severe hemorrhage ulcerated or actually regressed.

It is interesting to note that repeated intraperitoneal injections of 5 mg/kg of Shear's bacterial polysaccharide gave a marked inhibitory effect on the development of tumor ascites, but had no effect if it were given subcutaneously. Daily nontoxic doses of 5–10 mg/kg of the polysaccharide had no effect upon the growth of solid Ehrlich mouse carcinoma.

It is apparent from the results shown in Table 2 that the chemicals tested had a greater ability to inhibit the growth of the ascites tumor cells than the solid tumor. This is probably due to the more extensive direct contact of chemicals with tumor cells.

Our studies of compounds against a spectrum of tumors showed that tumor regression achieved with higher doses of certain compounds was accompanied by definite indications of toxicity, as evidenced by loss of weight and deaths. We do not, however, attribute the tumor inhibition by certain compounds with proper dose levels to general toxicity and weight loss. Nevertheless, the question of tumor inhibition due to underfeeding must always be considered as a possible indirect effect of administered drugs. This holds true also in the case of ascites tumor, as the following experiment shows. As a control for the compounds at dose levels causing loss of weight in the animals, we studied the effect of underfeeding upon the growth of Ehrlich ascites tumor.

Table 3 shows clearly that daily ingestion of 2–5 gm. of a normal diet (Purina Laboratory Chow) had no effect on Ehrlich ascites tumor, both solid and ascitic forms. However, when daily ingestion of food was reduced to about $\frac{1}{3}$ of normal requirements, the growth of ascites tumors was slightly diminished. The tumor-bearing mice on this diet lost body weight (0.35 gm. daily), and about 30 per cent of animals died during the 14 days. There was no tumor regression.

The study was extended with compounds such as nitrogen mustards, phosphoramides, purines, pyrimidines, triazines, steroids, protein hormones, antivitamins, antibiotics, and miscellaneous compounds which have unusual theoretical interest. Data for selected compounds at tolerated doses are shown in Table 4. The first intraperitoneal or subcutaneous injection of compounds was started 1 day after transplantation of the solid tumor or injection of the ascites tumor, and injections were continued for 7 days.

The table shows that repeated intraperitoneal or subcutaneous injections of HN2 oxide at the nontoxic level of 2 mg/kg had a marked inhibitory effect upon the development of ascites but no effect on solid tumors. As in the case of HN2, the oxide of HN2 had a destructive effect on well established ascites. Seven daily treatments with 2 mg/kg of HN2 oxide resulted in complete regression of ascites in about 50 per cent of cases (40

TABLE 4

Effect of Various Compounds on Ehrlich Mouse Carcinoma

Compound	No. of mice with tumors Solid form	Ascitic form	Dosage mg/kg/day	Route	Results of treatment
Methylbis(β-chloroethyl)amine oxide HCl	10		2	S.I.*	−
	10		2	I.P.†	−
		30	2	S.I.	++
		20	2	I.P.	++
Urethan	10		500‡	I.P.	−
		10	500‡	S.I.	−
		15	500‡	I.P.	±
N,N-Diethyl-N′,N″-diethylene-phosphoramide	10		4	I.P.	−
		15	10	I.P.	++
		15	6	I.P.	+
		20	4	I.P.	±
N,N′,N″-Triethylene phosphoramide	10		10	I.P.	+
	5		6	I.P.	±
		20	6	S.I.	±
		10	10	I.P.	++
		20	6	I.P.	++
6-Mercaptopurine hydrate	20		50	I.P.	++
		20	50	I.P.	++
		30	35	I.P.	++
		10	20	I.P.	++
8-Methyl xanthine		5	250	I.P.	−
2-Azaadenine	5		12.5	I.P.	−
		10	12.5	I.P.	−
2,4-bis(ethylenimino)-6-chloropyrimidine	20		2.5	I.P.	±
		30	2.5	I.P.	++
		10	2.5	S.I.	−
	10		2	I.P.	−
		10	2	I.P.	++
2-Ethylenimino-4-methoxy-6-chloro-pyrimidine		5	6	I.P.	+
2-Amino-4-methyl-*p*-(anisole) amino-6-methylpyrimidine		5	2	S.I.	±
2,4-Diamino-5-(3′,4′-dichloro-phenyl)pyrimidine	5		10	I.P.	−
		10	10	I.P.	−
2,4-Diamino-5-(3′,4′-dichloro-phenyl)-6-methylpyrimidine	10		4	I.P.	±
		5	4	I.P.	−
		5	2	I.P.	−
2,4-Diamino-5-(3′,4′-dichloro-phenyl)-6-ethyl pyrimidine	5		10	I.P.	±
		10	10	I.P.	−
1,2,3,6-Tetrahydro-2,6-dioxo-4-pyrimidine carboxylic acid		5	150	I.P.	−
2,4-Diamino-5-*p*-chlorophenyl-6,6-dimethyl-5,6-dihydro-1,3,5-triazine	5		35	I.P.	−
		5	35	I.P.	−
17-Hydroxy corticosterone acetate (Compound F)		15	37.5	S.I.	++
Allopregnane diol, 3β, 17α-one 20, acetate 3 (Compound L)	5		500	S.I.	−
		5	500	S.I.	−
Dexoxycorticosterone acetate	5		500	S.I.	−
		5	500	S.I.	−
17α-Hydroxy-3β-acetoxyallo-pregnane-20-one	5		500	S.I.	−
		5	500	S.I.	−
21-Acetoxy-allopregnane-21-ol-3,20-dione	5		250	S.I.	−
17-Methyl androstene diol	5		375	S.I.	−
3β-Hydroxy-16β-methyl-Δ^5-pregnene-20-one	5		500	S.I.	−
Pregnenolone	5		100	S.I.	−
		5	100	S.I.	−
Progesterone		5	500	S.I.	−
		10	375	S.I.	−
Allopregnane-3β-21-diol-20-one diacetate	5		375	S.I.	−
$\Delta^{1,17}$-Pregnadien-3β-ol	5		500	S.I.	−
Δ^4-Androstene-3,17-dione		5	500	S.I.	++
	10		375	S.I.	+
		5	375	S.I.	−
Testosterone propionate		10	500	S.I.	−
Δ^5-3-Hydroxy-20-cholenic acid lactone	5		500	S.I.	−
Estradiol benzoate		5	25	S.I.	−
ACTH		10	100‡	I.P.	±
	10		50§	S.I.	+
		10	50§	S.I.	++

* S.I. = Subcutaneous injection.
† I.P. = Intraperitoneal injection.
‡ Given in two doses.
§ Given in twelve doses.

TABLE 4—*Continued*

Compound	No. of mice with tumors: Solid form	No. of mice with tumors: Ascitic form	Dosage mg/kg/day	Route	Results of treatment
Diethylstilbestrol dipropionate		10	10	S.I.	−
4,6-Dimethyl-5-hydroxy-3-hydroxy-methyl pyridine hydrochloride	5		125	I.P.	+
		5	125	I.P.	−
Penicillin		20	1,500	I.P.	−
Streptomycin		20	500	I.P.	−
Aureomycin		15	50	I.P.	±
		10	25	I.P.	−
Chloromycetin		15	500	I.P.	−
Terramycin		25	150	I.P.	−
Borrelidin	5		2	I.P.	−
		5	2	S.I.	−
		5	1	S.I.	−
		15	4	I.P.	±
		15	3	I.P.	−
		15	2	I.P.	−
Diasone	5		500	I.P.	±
		5	500	I.P.	+
2,5-Dimercapto-1,3,4-thiodiazole		5	50	I.P.	−
2,4-Dinitrophenol		5	20	I.P.	+
1,1-Diphenyl-2,2-dimethyl propane	5		125	I.P.	−
		5	125	I.P.	−
Formamide	5		250 c.mm.	I.P.	+
		10	250 "	I.P.	±
N-methyl formamide	5		150 "	I.P.	−
		20	250 "	I.P.	±
		5	150 "	I.P.	−
Histamine		5	25	I.P.	−
		5	10	I.P.	−
Malononitrile	5		6	I.P.	−
		5	6	I.P.	−
Marsilid	5		175	I.P.	−
		5	175	I.P.	−
Merthiolate	10		10	I.P.	±
		5	10	I.P.	−
3′-Methyl-2,4-dinitro-diphenyl-amine		5	500	I.P.	−
Podophyllotoxin		10	2	I.P.	−

animals). Effectiveness of both mustards against the Ehrlich ascites tumor appeared the same.

Urethan had practically no inhibitory effect on Ehrlich mouse carcinoma, both in solid form and ascitic form.

N,N′,N″-triethylene phosphoramide (TEPA), which contains one more ethylenimine group than N,N-diethyl-N′,N″-diethylene phosphoramide (DEPA), is not much more effective than DEPA in Ehrlich ascites tumor. Thus, at the nontoxic level of 10 mg/kg of TEPA and DEPA there was a marked inhibitory effect on tumor ascites, while at 6 mg/kg, TEPA appears to be more effective than DEPA. Both compounds had practically no effect on solid tumors.

It is interesting to note that repeated intraperitoneal injections of 6-mercaptopurine had a marked inhibitory effect on Ehrlich mouse carcinoma, both in solid form and ascitic form. This tumor inhibitory action was first observed with Sarcoma 180 (4). Three other purine analogs tested showed no inhibitory effect.

Of seven pyrimidines, only 2,4-bis(ethylenimino)-6-chloropyrimidine had a marked inhibitory effect on ascites. Others had almost no effect on Ehrlich ascites tumors.

The results with cortisone encouraged study of numerous steroids on Ehrlich ascites tumor. At the maximum tolerated dose of 37.5 mg/kg/day, Compound F appears to be nearly as active as cortisone, whereas Compound L has shown no activity.

Repeated subcutaneous injections of twelve other steroids at tolerated doses had no inhibitory effect on Ehrlich mouse carcinoma, both solid form and ascitic form. These are: desoxycorticosterone acetate, 17α-hydroxy-3β-acetoxyallopregnane-20-one, 21-acetoxyallopregnane-3,20-dione,17-methyl androstene diol, 3β-hydroxy-16-β-methyl-Δ^5-pregnene-20-one, progesterone, allopregnane-3β-21-diol-20-one diacetate, $\Delta^{5,17}$-pregnadiene-3β-ol, pregnenolone, testosterone propionate, Δ^5-3-hydroxy-20-cholenic acid lactone, and estradiol benzoate.

A slight inhibition has been shown by ACTH in doses of 100 mg/kg/day twice daily. At one-

half the total dosage given every 2 hours for a week, there was a marked inhibition of tumor ascites. A quick elimination of the ACTH in the former case is probably responsible for its ineffectiveness.

Repeated injections of 4,6-dimethyl-5-hydroxy-3-hydroxymethyl pyridine hydrochloride, an antivitamin, had a moderate inhibitory effect on solid tumors but no effect on ascites.

We also tested antibiotics at high dosages on Ehrlich ascites tumor. Daily intraperitoneal injections of 1,500 mg/kg of penicillin, 50 mg/kg of streptomycin, 50 mg/kg of aureomycin, 500 mg/kg of chloromycetin, and 150 mg/kg of terramycin had neither inhibitory nor stimulating effects upon the development of Ehrlich ascites tumor in mice.

At the maximum tolerated dose, borrelidin had only a slight inhibitory effect; diasone and 2,4-dinitro phenol had a moderate inhibitory effect on tumor ascites; formamide[5] had a slight inhibitory effect, but 2,5-dimercapto-1,3,4-thiodiazole, 1,1-diphenyl-2,2-dimethyl propane, N-methyl formamide, histamine, malononitrile, marsilid, merthiolate, 3′-methyl-2,4-dinitro-diphenylamine, and podophyllotoxin had no inhibitory effect. These compounds were tested because of data obtained with Sarcoma 180.

All the effects described above are subject to possible inaccuracies of method. Klein (14) has discussed some of these problems, as well as the advantages of using the ascitic form of tumors. The ascites may also provide a good source of suspended tumor cells useful for biochemical studies. Chemotherapeutic effects may be readily observed histologically by sampling ascitic fluid at intervals during and after treatment. Quantitative evaluation of results in chemotherapy studies with ascitic tumors has not yet been placed upon as definite a basis as similar investigations with solid tumors. The formation of solid tumors at the site of injection, along with the ascites, may complicate the evaluation whether it is based upon weight of the mouse or upon increase in survival time. It has been suggested (14) that treatment with chemicals may decrease the amount of ascitic fluid without significantly influencing the number of tumor cells. In such cases this obviously could result in misleading information in the interpretation of weight measurements. The ascites tumor provides an easy challenge to chemotherapeutic agents because the intraperitoneal injection of compounds against the intraperitoneal ascitic form of tumors is in effect an *in vivo-in vitro* test.

[5] D. A. Clarke, F. S. Philips, S. S. Sternberg, and C. C. Stock. Formamide Analogs: Inhibitor of Mouse Sarcoma 180 (in preparation).

SUMMARY

1. The effects of nitrogen mustards, phosphoramides, purines, pyrimidines, triazines, folic acid analogs, pteridines, steroids, protein hormones, antivitamins, antibiotics, and miscellaneous compounds were studied on the growth of Ehrlich ascites tumor.

2. Repeated subcutaneous or intraperitoneal injections of HN2 or HN2 oxide had a destructive effect on the tumor ascites but none, or nearly none, on the solid form.

3. Triethylene melamine, triethylene phosphoramide, diethylene phosphoramide, and a methoxy pyridoxine mustard had a marked inhibitory effect on the development of tumor ascites when injected intraperitoneally.

4. Subcutaneous injections of cortisone had a destructive effect on tumor ascites, while intraperitoneal injections had but slight effect. Compound F appears to be nearly as active as cortisone, whereas other steroids tested have shown no activity on Ehrlich ascites tumor.

5. Intraperitoneal injections of 6-mercaptopurine had a marked inhibitory effect on Ehrlich mouse carcinoma, both in the solid and ascitic forms.

6. Repeated intraperitoneal injections of Shear's bacterial polysaccharide had a marked inhibitory effect on the development of tumor ascites, but had no effect if given subcutaneously.

7. Colchicine had a slight inhibitory effect on Ehrlich ascites tumor, but urethan and podophyllotoxin had no inhibitory effect.

8. 2,6-Diaminopurine, 8-azaguanine, and five pyrimidines tested had no inhibitory effect on Ehrlich mouse carcinoma, whether in solid or ascitic form.

9. Daily subcutaneous or intraperitoneal injections of folic acid, aminopterin, and A-methopterin had no inhibitory effects either on the solid or on the ascitic form of Ehrlich mouse carcinoma.

10. Repeated intraperitoneal injections of pure antibiotics, penicillin, streptomycin, aureomycin, chloromycetin, and terramycin had neither inhibiting nor stimulating effects upon the development of Ehrlich ascites tumor in mice.

11. The Ehrlich ascites tumor is a useful tool for testing the activity of chemicals.

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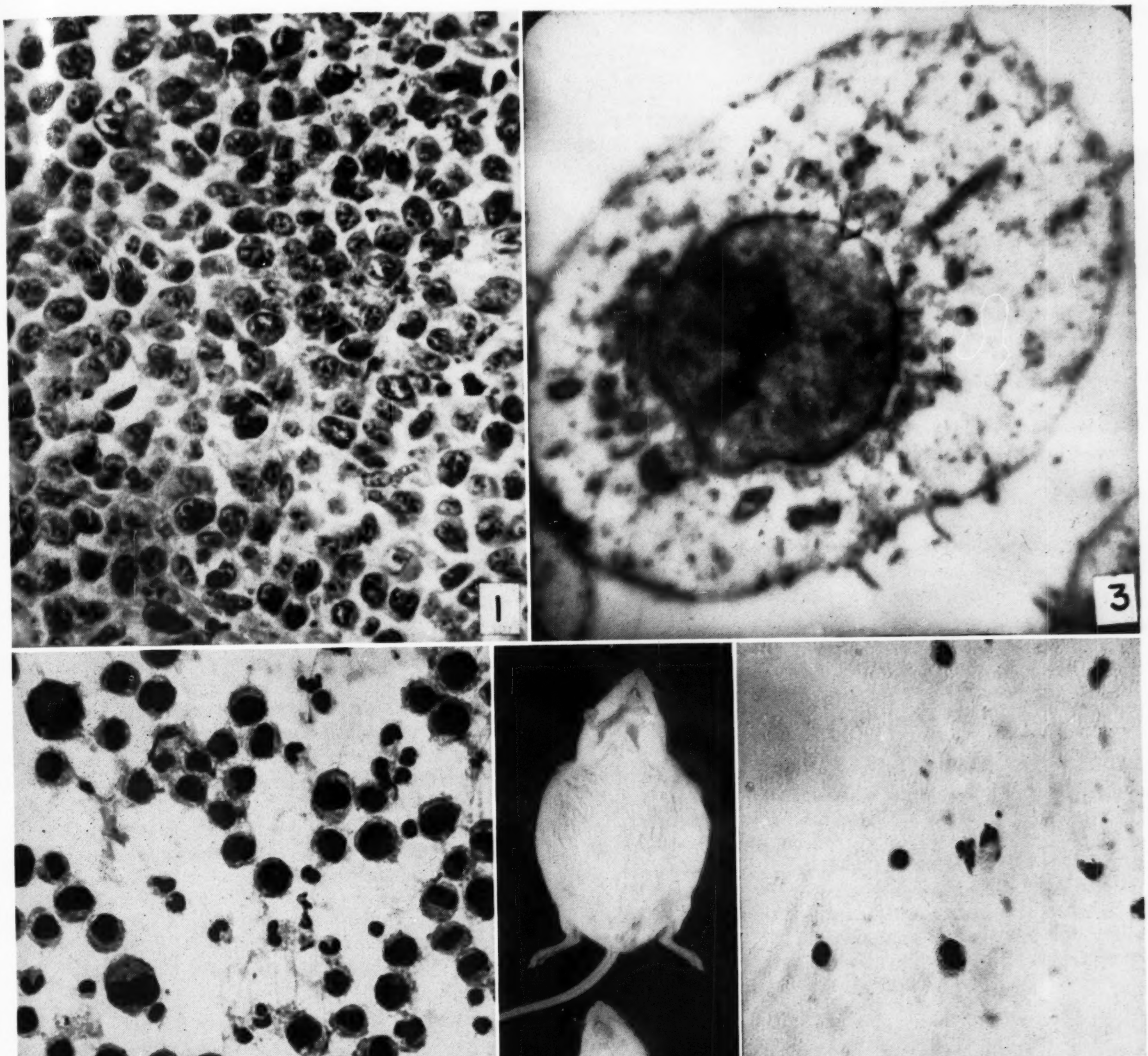
Fig. 1.—Histological picture of solid Ehrlich mouse carcinoma, showing solid sheets of rounded cells. (Hematoxylin and eosin stain. × 400.)

Fig. 2.—A general view of the smear of Ehrlich ascites tumor, showing an abundance of active cancer cells (large cells) and a small number of normal cells (small cells). (Papanicolaou stain. × 400.)

Fig. 3.—Electron micrograph of Ehrlich ascites tumor. (Osmic acid fixation. × 6,800.)

Fig. 4.—The top row illustrates control with marked distention of abdomen due to rapid growth of Ehrlich ascites tumor. The bottom row illustrates HN2-treated mouse (daily doses of 1.0 mg/kg of HN2 on 7 consecutive days). There is no distention of the abdomen, indicating absence of ascites. The animals were photographed 20 days after inoculation of cancer cells.

Fig. 5.—Ehrlich ascites tumor after treatment with HN2 (1.0 mg/kg for 7 days). The smear section shows very few cancer cells, and they are all degenerated. The small cells are normal cells, such as leukocytes, histiocytes, and mesothelial cells. (Papanicolaou stain. × 400.)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The Failure of Biotin or Oxybiotin To Exert a "Procarcinogenic" Effect on Tumor Formation by 4-Dimethylaminoazobenzene

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Oxybiotin, the oxygen analog of biotin (4, 7), possesses biotin-like activity for various microorganisms, the rat, and the chick (4, 8, 15, 17). In this respect, oxybiotin is unique among the vitamins of the B complex, since biological activity is retained in spite of the substitution of the sulfur of the ring by oxygen. It has been demonstrated that the activity of oxybiotin is an intrinsic property of the molecule and is not due to its conversion to biotin (1, 12, 13).

TABLE 1

Composition of the Basal Diet*

Constituent	Gm/100 gm of diet
"Vitamin-free" casein (Labco)	15
Primex	10
Sucrose	60
Egg white (dried)	10
L-cystine	1
Salt mixture (Osborne and Mendel)	4
Choline	0.25
Riboflavin	0.002
Thiamine	0.0005
Pyridoxine	0.0005
Nicotinic Acid	0.002
i-Inositol	0.100
Pantothenic acid	0.001
2-Methyl-1,4-naphthoquinone	0.0001
dl-α-Tocopherol acetate	0.001
4-Dimethylaminoazobenzene	0.100

* Each 100 grams of diet contained 4,070 units of Vitamin A and 814 units of Vitamin D supplied as Natola (Parke, Davis & Co.). 4-Dimethylaminoazobenzene was incorporated in the diet by dissolving it with heat in the Primex.

Du Vigneaud *et al.* (5) have reported that biotin possesses a procarcinogenic effect on tumor formation by 4-dimethylaminoazobenzene. Pursuant to our investigations on the biological activity of oxybiotin, we undertook a comparison of the procarcinogenic activity of this compound to that of biotin under conditions similar to those of du Vigneaud *et al.* (5). These studies, presented in this paper, failed to afford any evidence for the procarcinogenic activity of either oxybiotin or biotin.

METHODS

Male, weanling albino rats were obtained from the Sprague-Dawley colony and kept in individual, wide-meshed, screen-bottomed cages. During the first 4 weeks, the animals were fed Purina Dog Chow. They were then transfered to the purified basal diet[1] containing 4-dimethylaminoazobenzene (Table 1), which, for some groups, was supplemented with biotin or oxybiotin as described in the following section. The diets, which were fed ad libitum for the duration of the experiment, were freshly prepared every 2 weeks and stored in the refrigerator. The animals were weighed at frequent intervals, and their daily food intakes determined periodically. At the conclusion of the experiment, the surviving rats were sacrificed by decapitation.

Upon death of the animal, a portion of the liver and, when present, of liver tumor was removed and fixed in formalin for histologic studies. The remainder of the liver and liver tumor was dried at 45° C. *in vacuo* over phosphorus pentoxide. The thoroughly ground tissue (500 mg.) was autoclaved at 15 lbs. for 90 minutes with 15 ml. of 4 N sulfuric acid and filtered. After neutralization with sodium hydroxide, the biotin content of the filtrates was determined by the procedure of Wright and Skeggs (19). A differential assay for the biotin and oxybiotin contents was conducted by the Raney's nickel method of Hofmann *et al.* (9). Care was taken to utilize only the grossly nonnecrotic portion of the liver tumor for both histologic examination and biotin analysis.

RESULTS

In a preliminary experiment extending for 8 months, twenty rats received the basal diet, while an equal number received the same diet supple-

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[1] Patterned after diet B of du Vigneaud *et al.* (5).

mented with 40 μg of *d*-biotin/100 gm. No hepatic tumors were present in either group, as determined by gross or microscopic examination. However, significant histopathologic findings in the liver, similar to those noted in experimentally induced cirrhosis, were observed in four animals of each group. The degree of histopathologic change was comparable in the two groups.

Since it seemed probable that the histologic changes observed in the above study were preliminary to the development of hepatic tumors, the experimental period during which the diets containing 4-dimethylaminoazobenzene were fed was extended to 12 months in the subsequent investigation. Sixty animals were distributed into three equal groups (Table 2). One group received only the basal diet, while the remaining two groups received the basal diet plus 40 μg of *d*-biotin and 800 μg of *dl*-oxybiotin/100 gm, respectively. This quantity of *dl*-oxybiotin was used, since previous investigations had shown that *dl*-oxybiotin is approximately 5 per cent as effective as *d*-biotin in curing biotin deficiency in the rat (2).

A moderate biotin deficiency developed in the animals receiving only the basal diet, as evidenced by their decreased growth (Table 2) and liver biotin contents (Table 3), as well as by the appearance of a slight degree of alopecia, dermatitis, and cheilosis. The rats fed biotin and oxybiotin-supplemented diets did not show these symptoms. No significant differences in the daily food intakes of the animals in the various groups were observed. The average daily food intake was 14 gm/rat.

The hepatic tumor incidence in the various groups is shown in Table 2. The gross observations were confirmed by microscopic examination. In some animals of each group the tumor resembled a hepatoma, while in others there was obvious adenocarcinoma. In addition, the histopathologic changes observed in the preliminary study were also present in the livers of many animals in this series. These changes were distributed uniformly among the three groups.

The results of the biotin and oxybiotin analyses of livers and hepatic tumors are given in Table 3.

TABLE 2

THE EFFECT OF BIOTIN AND OXYBIOTIN UPON 4-DIMETHYLAMINOAZOBENZENE CARCINOGENESIS

Group	No. of rats	Av. weight* Initial (gm.)	Final (gm.)	Cumulative hepatic tumor incidence at: 8 mo.	9 mo.	10 mo.	11 mo.	12 mo.	Negative survival†
Basal	20	212	329	0	1	2	3	5	15/17
Basal+biotin	20	218	411	0	1	1	3	5	15/17
Biotin+oxybiotin	20	219	420	0	1	2	4	7	13/15

* Weights at beginning and end of the 12-month period on the 4-dimethylaminoazobenzene-containing diets.

† No. of rats surviving at 12 months with no hepatic tumors over total no. surviving at 12 months. All rats which succumbed prior to the termination of the experiment possessed hepatic tumors.

TABLE 3

BIOTIN AND OXYBIOTIN CONTENT OF LIVERS AND HEPATIC TUMORS*

Group	Liver† No. samples	*d*-biotin	*dl*-oxybiotin	Hepatic tumor No. samples	*d*-biotin	*dl*-oxybiotin
Basal	19	1.3 (0.7–1.7)	0	4	0.09 (0.03–0.13)	0
Basal+biotin	19	2.4 (2.0–3.0)	0	4	0.38 (0.33–0.45)	0
Basal+oxybiotin	19	1.0 (0.6–2.0)	2.9 (1.5–3.8)	7	0.19 (0.10–0.26)	0.27 (0.11–0.46)

* All values expressed as μg/gm dry weight. Range of values is given in parentheses.

† In some instances, analyses were made upon grossly normal liver tissue contiguous to a tumor. Values for such tissues did not differ from those of nontumor-bearing livers in the same group.

DISCUSSION

In reviewing the experiences of the Wisconsin group with 4-dimethylaminoazobenzene, Miller (14) reported a liver tumor incidence of 90–100 per cent at 6 months in rats of the Sprague-Dawley strain receiving certain nonprotective diets. The protective nature of the basal diet employed in the present experiments is evidenced by the extended latent period and lowered incidence of tumor formation. Thus, no tumors were present at 8

months, and at 12 months the tumor incidence was only 25–35 per cent. These results are in agreement with those of du Vigneaud *et al.* (5) and of Burk *et al.* (3), who, utilizing a basal diet practically identical with that employed in our studies, also found no hepatic tumors at 6 months. However, these workers noted a tumor incidence of 60 per cent at 6 months when the basal diet was supplemented with biotin. Despite attempts to reproduce the experimental conditions of du Vigneaud *et al.* and of Burk *et al.*, this procarcinogenic effect of biotin was not demonstrable in our experiments. Oxybiotin was also devoid of any significant procarcinogenic activity. The evidence for the importance of biotin in carcinogenesis is conflicting. While several workers have observed a procarcinogenic effect (3, 5, 6), others have presented data which lend no support for a particularly significant role of biotin in tumor formation (10, 11, 16, 18).

The lowered liver biotin content of the basal group is in agreement with the findings of Burk *et al.* (3). The correlation noted by these workers between the protection against hepatic tumor formation and the decreased liver biotin was not found in our experiments, where the tumor incidence did not parallel the liver biotin content. Miller (14) was unable to correlate the liver biotin content with the protective character of various 4-dimethylaminoazobenzene-containing diets.

As seen in Table 3, the biotin content of the hepatic tumors produced by the feeding of 4-dimethylaminoazobenzene was significantly lower than that of liver tissue. A similar observation has been made by other investigators (3, 10, 14, 18). It is of interest that supplementation of the basal diet with biotin markedly increased the biotin content of the tumors (Table 3) without affecting either their incidence, size, or histopathology. West and Woglom (18) have similarly observed a lack of correspondence between the biotin content and growth of rapidly growing transplants of Sarcomas 37 and 180.

CONCLUSION

1. No evidence has been obtained for a "procarcinogenic" action of *d*-biotin or *dl*-oxybiotin in a protective diet containing 4-dimethylaminoazobenzene.

ACKNOWLEDGMENTS

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Hypercalcemia, a Complication of Hormone Therapy of Advanced Breast Cancer*†‡

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Steroid hormones and castration have been successfully employed in the palliative treatment of advanced mammary cancer (3, 8, 9, 12, 16, 17, 24, 29–31, 35).[1] Complications may arise during this therapy, however, which necessitate special consideration; one of the more serious of these is hypercalcemia. The progressive advanced stages of the syndrome are manifested by anorexia, nausea, vomiting, apathy, weakness, drowsiness sometimes merging into disorientation, stupor or coma, vascular collapse, and in some instances, death (1, 2, 9, 13, 18–22, 32, 34). Associated with these symptoms are an elevation of the serum and urinary calcium, frequently electrolyte changes, and sometimes renal insufficiency.

The present report records the changes observed in nine patients with obvious hypercalcemia culled from a series of 361 women with advanced cancer of the breast who were treated with sex steroid hormones. The complication in this series occurred only in patients with osseous involvement. Eighty-four of 253 patients treated with estrogens and 97 of 108 treated with androgenic hormones demonstrated osseous metastases. Of these, seven patients treated with androgens and two patients treated with estrogens developed profound manifestations of the hypercalcemia syndrome. Two patients with hypercalcemia, not included in the above consecutive series, were studied recently and have been incorporated in this report. In addition, a number of patients developed clinical manifestations compatible with a slight and transient hypercalcemia. Since these were usually of short duration, laboratory evidence of hypercalcemia was unavailable or inconclusive.

The following are case reports of eleven patients with osseous metastases secondary to breast cancer who developed hypercalcemia confirmed by laboratory examination during the course of steroid therapy.

Case I

L. B., age 63. M.G.H. #404392 (Charts 1 and 2). This woman was admitted to the Metabolic Ward of the Massachusetts General Hospital on January 8, 1948, for study and treatment of recently obvious manifest metastases from a breast cancer treated 5 years previously by radical mastectomy. She complained of weakness and loss of weight. Examination revealed a 5.5 × 4.5 cm. raised, fixed mass above the left eye invading the orbit and displacing the globe downward, partially impairing vision; a moderate degree of ptosis of the upper lid and limitation of upward gaze; a 3 × 2.5 cm. raised hard mass in the right parietal area near the midline and two firm 0.5 cm. lymph nodes in the left supraclavicular area. There was no evidence of recurrence in the operative field. The remaining physical examination was noncontributory. Her weight was 53 kg.

Pertinent blood studies were: hemoglobin, 13.0 gm.; serum calcium, 10.5 mg. per cent; serum phosphorus, 3.8 mg. per cent; NPN (nonprotein nitrogen), 26 mg. per cent; and total protein, 5.9 gm. per cent.[2] Liver function studies, phenol sulfon-

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† The testosterone propionate was generously supplied by the Ciba Pharmaceutical Products, Inc. (Perandren) and the Schering Corporation (Oreton).

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[1] I. T. Nathanson and B. J. Kennedy, unpublished data.

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[2] Normal blood chemistry values at the Massachusetts General Hospital and Pondville Hospital:

Serum calcium	9–10.8	mg. per cent
Serum phosphorus	2.5– 3.8	mg. per cent
NPN	15–35	mg. per cent
Total protein	6– 8	gm. per cent

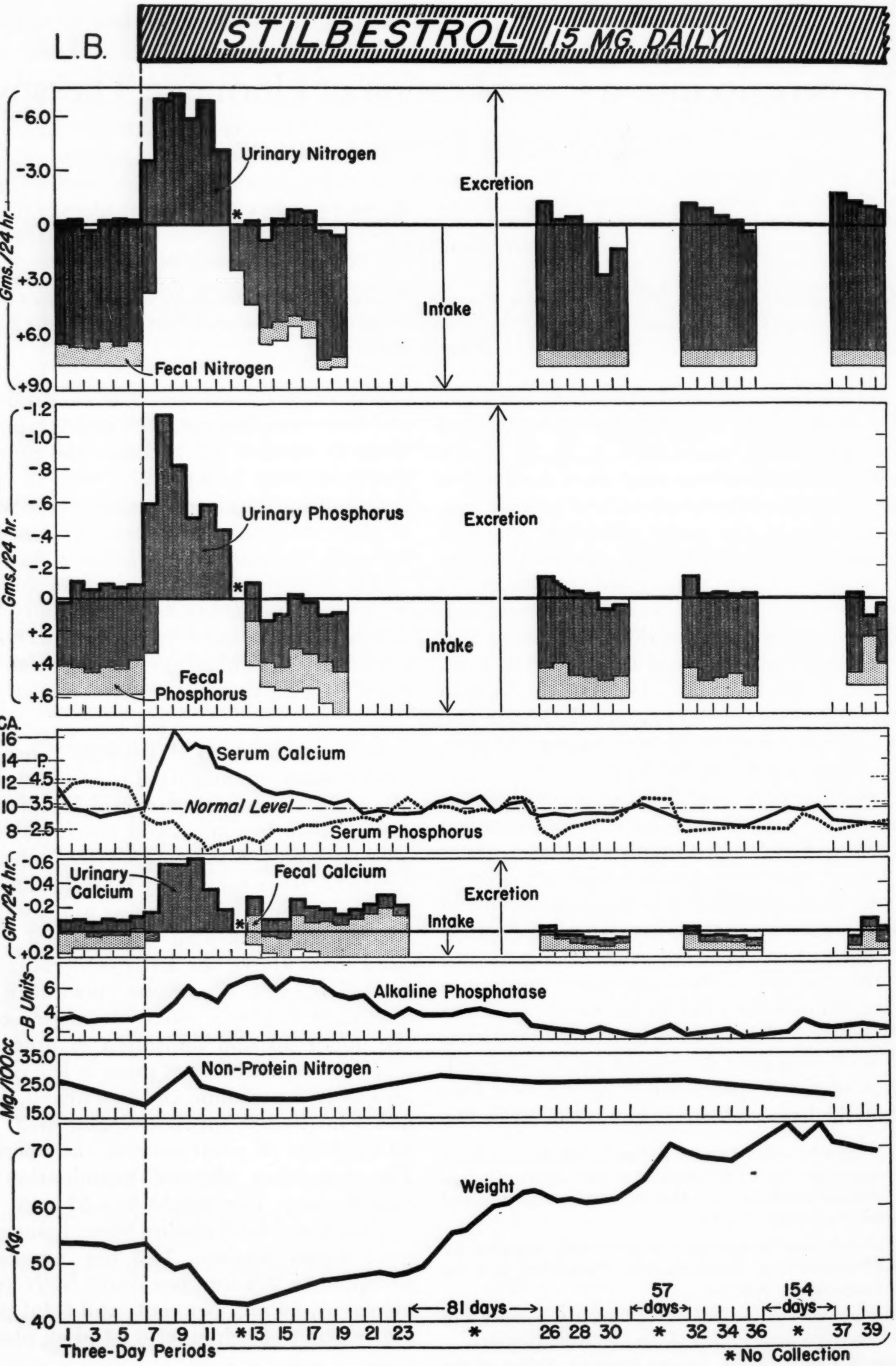

Chart 1.—Case I: Hypercalcemia due to stilbestrol, showing serum chemistries and balance data for calcium, phosphorus and nitrogen. The daily intake is charted from the 0 line downward, and the average daily excretion from the bottom line upward. A negative balance is therefore indicated by extension of the column above the line; and a positive balance by a clear area below the 0 line.

phthalein (PSP) excretion test, and urinalysis were within normal limits. Radiographs revealed a 5 × 4 cm. osteolytic lesion in the right parietal bone and a 4 × 3 cm. defect in the left frontal bone. There were dense, lobular, metastatic infiltrations in the lungs below the right hilus and overlying the arch of the aorta. A biopsy of the mass of the left forehead was consistent with metastatic carcinoma of the breast.

The patient was placed on a constant, neutral ash, low calcium (135 mg.) diet. Metabolic studies were commenced after an interval of 6 days to insure stabilization of the patient on the diet. Urines and stools were collected in 3-day pools cent. The serum protein had risen from 5.9 to 7.3 gm. per cent, but the NPN had decreased to 19 mg. per cent. On the fourth day the serum calcium was 13.4 mg. per cent; serum phosphorus, 2.7 mg. per cent; and an indirect bilirubin was 2.8 mg. per cent. By the 6th day vomiting ceased, but pain in the back developed. The mass invading the left orbit appeared to further displace the left eye downward, and diplopia occurred. The serum calcium had risen to 16.7 mg. per cent. The condition of the patient became precarious. The diet had to be discontinued, and adequate stool collections for metabolic studies were not possible. In spite of little or no intake of food, the urinary

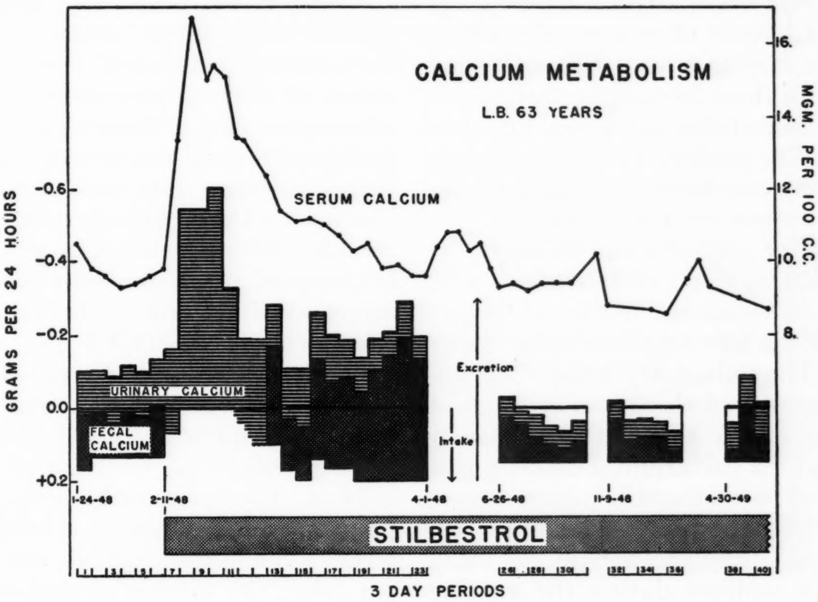

CHART 2.—Case I: Calcium balance and serum calcium (from Chart 1), revealing the hypercalcemia and hypercalcuria at the onset of estrogen therapy, followed by a positive calcium balance several months later. The rise of serum calcium between balance studies was due to a high calcium intake while the patient was at home.

throughout the study, except as indicated. The essential components were calculated in order to determine the metabolic balance. Pretreatment control studies were carried out for 18 days, divided into six periods of 3 days each. The patient was ambulatory. During the control period, the studies indicated a nitrogen equilibrium, negative calcium and phosphorus balances, and essentially constant blood levels (Chart 1).

Estrogenic therapy, diethylstilbestrol, 15 mg. daily, was instituted at the conclusion of the control period on February 11, 1948. That evening, the patient was unusually drowsy, nauseated, and vomited several times. Since this persisted for the ensuing 3 days, the patient remained in bed. There was no change in the serum calcium the first morning after medication, but the serum phosphorus value was reduced from 4.1 to 3.0 mg. per excretion of both calcium and phosphorus was considerably increased, and the pretreatment level of urinary nitrogen excretion was maintained. The highest urinary excretion of calcium, 670 mg. in 24 hours, was obtained on the 7th day of medication (Chart 1). Disorientation developed at this time. Intravenous glucose and saline administration, because of dehydration, was followed by improvement in orientation and appetite. Estrogen therapy was continued because of this suggested improvement. For the next 5 days the urinary and serum calcium remained high, with a serum calcium level of 15.1 mg. per cent and a total protein of 5.9 gm. per cent. During this time the phosphorus excretion in the urine diminished to the control level, and the serum phosphorus reached its lowest value of 1.6 mg. per cent. There was also a slight but definite rise in the serum

alkaline phosphatase. On the 16th day, the serum and urinary calcium had decreased, and the serum indirect bilirubin was 0.87 mg. per cent. The PSP excretion test, serum total protein, and NPN remained normal.

Twenty-five days after the onset of estrogen therapy, the drowsiness had disappeared, and the serum calcium had descended to 11.4 mg. per cent on continued stilbestrol therapy. In the succeeding 2 weeks the back pain diminished. Definite regression of the metastatic lesions was obvious by the 40th day of treatment. The mass over the left eye was flat, and a considerable decrease in size of the pulmonary lesions was observed. A biopsy of the mass at the vertex of the skull on the 50th day revealed scattered islands of cancer cells within edematous-looking fibrous tissue. These findings were consistent with those frequently observed in lesions of other patients following successful estrogen therapy (12). The patient was discharged on the 60th day of therapy because of marked improvement. The therapy was continued.

Four months after commencing therapy, the masses over the left eye and right parietal area were not discernible, vision and motion of the eye were normal, and the left supraclavicular nodes were impalpable. The pulmonary lesions had apparently disappeared, and there was evidence of calcium deposition at the site of the previously destructive areas of the calvarium. Further metabolic studies demonstrated that the patient was in positive calcium, phosphorus, and nitrogen balance. Following this study the patient remained well and free of symptoms during the ensuing 5 months.

The patient was admitted for a third metabolic study, 9 months after the onset of therapy. Examination revealed still further regression of the metastatic deposits. The lesions in the calvarium were depressed, hard, and appeared to be partially calcified. The patient remained in positive calcium balance and in nitrogen and phosphorus equilibrium. A biopsy of the parietal lesion of the skull at this time demonstrated new bone formation near the dura with residual islets of cancer cells. Tumor cells had apparently disappeared from the outer table of the skull.

During the ensuing several months, a weight of 74.5 kg. was attained (a gain of 31.5 kg. since the initial episode of hypercalcemia). There was no further apparent change in the disease until 1 year after the onset of continuous estrogen therapy. The original lesion in the left frontal region became reactivated and continued to increase during the succeeding 2 months. X-rays revealed new destruction of the frontal bone surrounding the calcified area in the original left orbital lesion and of the floor of the anterior cranial fossa. Another osteolytic lesion involved the crest of the left ilium. There was recurrence of back pain. Finally, a fourth metabolic study 14 months after the onset of therapy demonstrated a slight negative calcium balance. During the 60th week of stilbestrol therapy, testosterone propionate, 100 mg. intramuscularly, 3 times weekly, was added. During 2 months of this combined therapy, the disease continued to progress. Stilbestrol was discontinued, and testosterone was omitted 1 month later. The patient progressively failed and died 21 months after the date stilbestrol therapy was commenced.

Comment.—Symptoms of hypercalcemia began almost immediately after estrogenic hormone therapy was instituted. First, there was an increase of urinary phosphorus and a decrease of serum phosphorus. Shortly thereafter, a rapid and profound rise in serum and urinary calcium occurred within a short period. Continued nitrogen loss with a significant decrease in intake was accompanied by a decrease in weight. There was no evidence of renal insufficiency, despite the high excretion of calcium in the urine. The metabolic effects closely resembled those produced by the administration of parathyroid extract, even to the sequence in which they occurred: (*a*) increased urinary phosphorus, (*b*) fall of serum phosphorus, (*c*) rise of serum calcium, and (*d*) increased urinary calcium. Though parathormone lowers the phosphorus level of blood of normal patients, if the serum calcium rises above a critical level of 14–15 mg., then the urinary phosphorus decreases and the serum phosphorus rises. Furthermore, the effect of parathormone in some cases gradually wears off, and the first evidence of this is a decreased phosphorus elimination (4). Similar alterations in metabolism were observed in this patient. The situation in this patient is of especial interest in that hypercalcemia and strongly negative calcium, phosphorus, and nitrogen balances, apparently initiated by stilbestrol, eventually were reversed with accompanying clinical improvement during continuation of the hormone. The rise in alkaline phosphatase preceding the reversal may be of significance in predicting the response of other patients.

Case II

M. T., age 56. P.H. #27446 (Chart 3). This patient was admitted to the Pondville Hospital 1 year after a right radical mastectomy for carcinoma, because of pain from an osteolytic lesion in the sixth thoracic vertebral body. She was ambulatory. The serum calcium was 11.8 mg. per

cent. On March 27, 1949, intramuscular injections of 100 mg. of testosterone propionate were given 3 times a week. The hormone was omitted 6 days later after the third injection because of vomiting. The serum calcium was 12.8 mg. per cent. On April 7, stilbestrol, 15 mg. daily, was started, but was discontinued after 4 days because of nausea. By April 14, the serum calcium was 18.0 mg. per cent, but fell to a normal level 2 days later. Estrogen therapy was then resumed. There was no change in the calcium values in the ensuing 2 months, and the patient continued to receive therapy for partial relief of pain. After 5 months of estrogen therapy, the patient omitted the medication because of nausea. During the subsequent 15 months there was a gradual progression of the disease until her death on December 16, 1950.

Comment.—This case demonstrated that transient hypercalcemia may occur at the onset of hormone therapy. The frequent nausea and vomiting and occasional drowsiness observed during the early courses of estrogen therapy may be a manifestation of this syndrome in some patients. There is some evidence that similar states, although

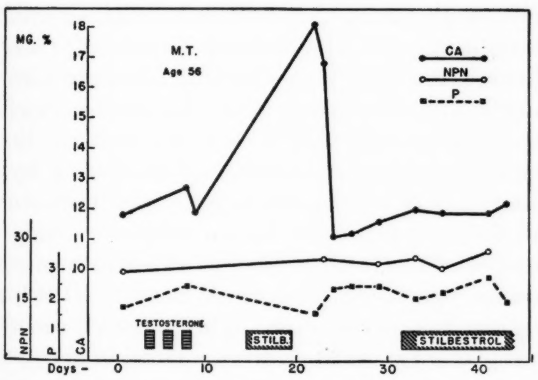

CHART 3.—Case II: Stilbestrol-induced hypercalcemia that disappeared soon after discontinuance of the hormone. Another trial of this hormone did not result in further complications.

more transient and less profound than those observed in these first two cases, may occur undetected in other women with comparable disease receiving steroid hormones. This demonstrates the necessity of close observation of these patients and of frequent serum calcium determinations.

CASE III

J. W., age 65. M.G.H. #622418 (Chart 4). This woman was admitted to the Metabolic Ward of the Massachusetts General Hospital in June, 1948, because of an inoperable, primary carcinoma of the right breast with probable extensive osseous metastases. There were anorexia, weakness, weight loss, and back pain. The patient was placed on a constant low calcium (135 mg.) diet. Metabolic studies similar to those in Case I revealed positive nitrogen and phosphorus balances and calcium equilibrium. The initial serum calcium was 8.9 mg. per cent; serum phosphorus, 3.9 mg. per cent; NPN, 21 mg. per cent; and total protein, 6.1 gm. per cent. X-rays suggested diffuse osteoporosis, but an aspiration of the bone marrow demonstrated

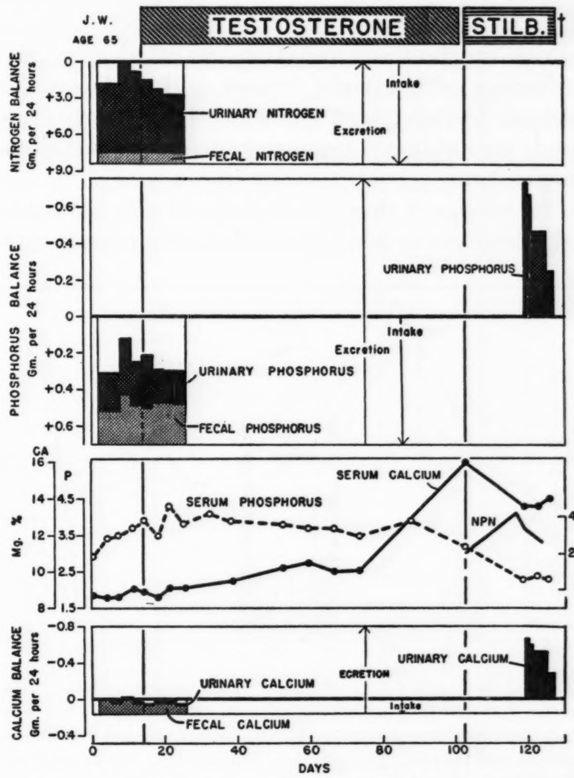

CHART 4.—Case III: At the onset of androgen therapy the patient was in positive nitrogen, phosphorus, and calcium balance. Hypercalcemia due to testosterone was followed by negative calcium and phosphorus balance but no gross renal insufficiency.

neoplastic cells. Testosterone propionate, 100 mg. 3 times weekly intramuscularly, was commenced in July, 1948. In the ensuing 60 days, there were an increase in appetite, slight weight gain, and partial relief of pain without obvious change in the demonstrable lesions. This temporary respite was followed by an increase of back pain, increase in size of the primary breast lesion, and an extension of osteolytic metastases. Because of this progression, androgen therapy was discontinued on the 87th day, and 15 mg. of stilbestrol daily was commenced the same day. At this time, the serum calcium was 16.0 mg. per cent; serum phosphorus, 3.2 mg. per cent; total protein, 6.7 gm. per cent; and the NPN, 21 mg. per cent (Chart 4). The patient was semi-ambulatory. Ten days later drowsiness and weakness occurred, followed by

anorexia and nausea. During the subsequent 10 days the stilbestrol was continued, but the patient became stuporous and incontinent of urine and feces. There was a marked increase in the calcium and phosphorus excretion; the serum calcium was 14.0 mg. per cent; serum phosphorus, 2.4 mg. per cent; NPN, 26.0 mg. per cent; and the van den Berg, 2.5 mg. per cent. Stilbestrol was omitted after 28 days of therapy. The patient died 2 days later.

Post mortem examination revealed extensive carcinoma of the right breast and skeleton. No cerebral lesions were present. The parathyroid glands and kidneys appeared normal on histologic examination.

Comment.—A delayed and essentially asymptomatic hypercalcemia occurred during testosterone therapy. The clinical status of the patient paralleled the chemical findings. The hypercalcemia and associated clinical findings may have been precipitated by testosterone therapy or may have occurred spontaneously. A shift in therapy to stilbestrol at the time of rapid progression of the process and hypercalcemia may have accentuated the disease and in turn contributed to the final result. On the other hand, it is conceivable that the established course of the disease was unaltered.

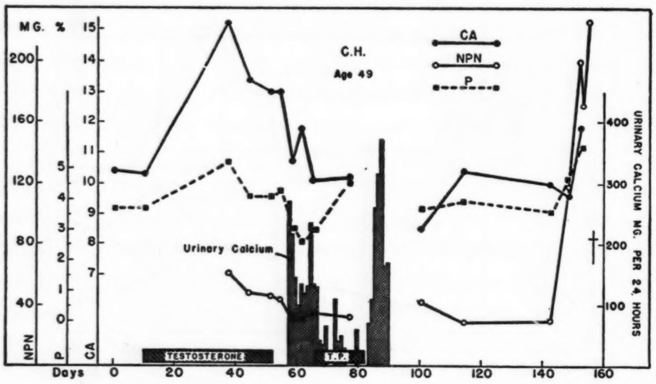

CHART 5.—Case IV: The initial course of testosterone propionate was uneventful. A second and third trial of the hormone (testosterone propionate) was accompanied by hypercalcemia and uremia. The terminal episode was due to obstruction of the ureters by cancer and resultant uremia.

CASE IV

C. H., age 48. M.G.H. #52707 (Chart 5). One month after a left radical mastectomy in May, 1946, osteolytic metastases were detected in the left ilium. Irradiation of the ovaries was followed by relief of pain and apparent restoration of normal bone in the involved area for 15 months before the reappearance of symptoms.

Reactivation of the disease, evidenced by extensive osteolytic metastases, accompanied by severe back pain, weight loss, and debility became obvious in September, 1947. The patient became bedridden. Testosterone propionate was commenced at a dose level of 50 mg. intramuscularly, 3 times weekly. Complete relief of pain and rehabilitation occurred within 2 months. Therapy was discontinued after 5 months, since the clinical status continued constant and many bone lesions had calcified. Since the disease remained stationary, an attempt to obtain calcification of remaining osseous lesions was instituted by a second course of testosterone propionate, 50 mg. 3 times weekly, 4 months later. At this time the serum calcium was 10.3 mg. per cent and serum phosphorus, 3.7 mg. per cent. Two weeks later the patient was forced to bed because of anorexia and nausea. Vomiting began during the 4th week. X-rays then revealed an increase in the number and size of destructive bone lesions. The serum calcium was 15.2 mg. per cent, with accompanying hypercalcuria; serum phosphorus, 5.2 mg. per cent; NPN, 61 mg. per cent; and the total proteins 7.1 gm. per cent (Chart 5). The specific gravity of the urine was 1.010. The PSP excretion was 25 per cent in 2 hours. Since nausea and vomiting became increasingly severe, testosterone was omitted on the 42d day of the second course of therapy. Within 10 days, there was a disappearance of nausea and vomiting, and the serum calcium, phosphorus, and NPN fell to normal limits. Urinary studies revealed a diminishing hypercalcuria. The total protein remained between 6.2 and 6.6 gm. per cent. In an effort to study the hypercalcemia, a third course of testosterone propionate was attempted on August 11, 1948, employing the same dose. The patient remained asymptomatic until 2 weeks later, when severe nausea and vomiting recurred, and the hormone was again discontinued with relief of the gastrointestinal symptoms. The serum calcium just prior to omission of the hormone was normal, but the serum phosphorus had risen again to 4.6 mg. per cent. Through an error, serial serum calcium determinations were not made in an ensuing period, but studies of the urinary calcium excretion revealed significant elevations (Chart 5). During the succeeding 7 weeks, the patient was relatively free of pain but had recurrent and increasing nausea, vomiting, epigastric cramps, weakness, and weight loss. The 8th week after cessation of hormone therapy, a large nodular liver and large nodes were palpated in the abdomen and pelvis. The serum calcium increased to 11.8 mg. per cent, but the serum phosphorus and NPN rose rapidly to high levels. The patient died in uremia, November 9, 1948.

Post mortem examination revealed extensive metastatic cancer in the skeleton, liver, pelvic viscera, and abdominal lymph nodes. The disease produced complete obstruction of the ureters, with associated hydronephrosis. On histologic examination, scattered speckles of calcium deposits were seen in the renal tissues. The parathyroid glands appeared normal.

Comment.—Hypercalcemia did not occur during the initial course of androgen therapy, but was manifested during the second course of therapy and repeated in a third trial of the hormone. Evidence of temporary renal insufficiency during the hypercalcemia consisted of elevation of the nonprotein nitrogen and inadequate PSP excretion. Though death was due to renal failure with terminal uremia, this apparently was the result of extensive pelvic metastases producing obstruction of both ureters. The calcium deposits in the kidneys secondary to hypercalcuria, in this instance, may have been a contributory cause of the renal insufficiency.

CASE V

A. F., age 61. M.G.H. #192478 (Chart 6). Two months after a left radical mastectomy, this woman developed back pain from osteolytic metastases in the spine. Diethylstilbestrol, 10 mg. daily, was started in December, 1946, and continued for 6 months. The pain decreased by the second month and was completely relieved by the fourth month. X-rays at this time revealed scattered calcifying bone lesions in the pelvis. However, during the 6th month of estrogen therapy, because of recurrent back pain and an increase in size of persistent osteolytic lesions in the spine and the appearance of additional osseous disease, stilbestrol was discontinued. Testosterone propionate, 50 mg. 3 times weekly, intramuscularly, was commenced immediately. The serum calcium was 8.6 mg. per cent; the serum phosphorus, 3.5 mg. per cent; and the NPN, 30 mg. per cent. Within 2 weeks the patient experienced relief of pain, an increased feeling of well-being, an increased appetite, and weight gain. After the 4th month the testosterone was omitted, since the patient was asymptomatic and the disease appeared static. The lesions remained unchanged on x-ray examination. During the ensuing 3 months the patient remained relatively comfortable, but excruciating pain recurred during the 4th month, and a second course of androgen therapy was started January 20, 1948. The serum calcium was 10.3 mg. per cent, and serum phosphorus, 4.7 mg. per cent. Two weeks later the patient developed moderate anorexia, nausea, vomiting, and abdominal distention. On the 18th day of the second course of therapy, the serum calcium (13.1 mg. per cent) and serum phosphorus (5.0 mg. per cent) were elevated (Chart 6). Vomiting and nausea had subsided by the 23d day of uninterrupted testosterone therapy. The serum calcium had returned to normal (calcium, 10.1 mg. per cent; phosphorus, 3.6 mg. per cent) by the 65th day of treatment. However, x-rays again revealed marked progression in the size and number of osteolytic metastases, and on the 79th day the nausea, vomiting, and back pain recurred and increased. On the 90th day of treatment, since the serum calcium was 15.2 mg. per cent, the testosterone was omitted. The patient was admitted to the hospital 7 days later, at which time the symptoms had subsided. Laboratory studies were:

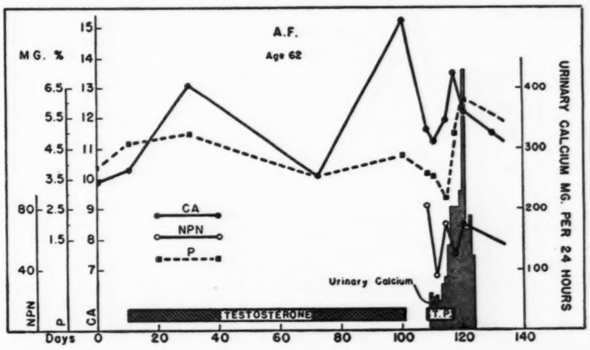

CHART 6.—Case V: Hypercalcemia occurring during a second and third course of testosterone propionate (T. P.).

serum calcium, 11.6 mg. per cent; NPN, 82 mg. per cent; specific gravity of the urine, 1.010 with slight albuminuria. The patient was immediately placed on a low calcium diet (150 mg/day), and testosterone propionate, 50 mg. given intramuscularly every other day, was commenced again, in order to study the metabolic phenomena. During the succeeding 9 days, the patient received five injections of the hormone. Anorexia, nausea, and vomiting increased steadily accompanied by a rise in serum calcium to 13.5 mg. per cent, phosphorus to 5.1 mg. per cent, and NPN to 70 mg. per cent, and a threefold increase in urinary calcium. Testosterone was discontinued. Four days later, in spite of a continued rise in the urinary calcium excretion and the serum phosphorus level to 6.1 mg. per cent, the serum calcium began to decrease toward normal values. The nausea disappeared several days later. Following the last episode of hypercalcemia, the patient remained bedridden, though relatively free of pain for 13 months, until death occurred from progression of the cancer. The serum calcium and phosphorus

remained normal, and the NPN varied from 30 to 78 mg. per cent.

Comment.—Though in the initial course of therapy the patient remained free of complications, the second and third courses of testosterone were accompanied by hypercalcemia. The occurrence of hypercalcemia and increased clinical manifestations, during the subsequent courses of testosterone therapy and partial recovery after discontinuance, suggest again that these symptoms are due to the blood calcium level rather than to increased growth rate of the tumor, about which we have no evidence.

Case VI

D. F., age 56. P.H. #26605 (Chart 7). This incapacitated woman was seen on August 1, 1948,

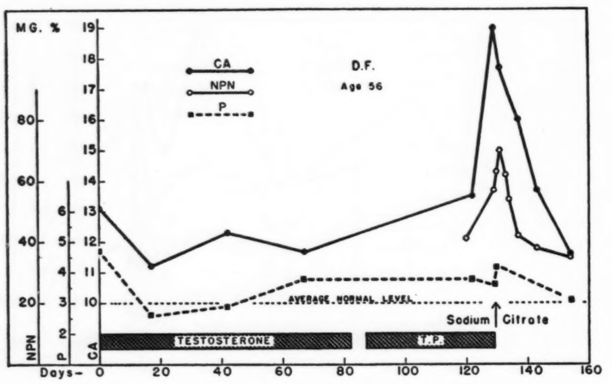

Chart 7.—Case VI: Hypercalcemia associated with temporary renal insufficiency improved slowly following sodium citrate infusion and discontinuance of androgen therapy.

because of severe pain in the legs and back due to extensive osteolytic metastases from a cancer of the left breast. A spontaneous hypercalcemia due to extensive osseous metastases was evidenced by a serum calcium of 13.1 mg. per cent and serum phosphorus of 4.7 mg. per cent. Testosterone propionate, 100 mg. intramuscularly 3 times a week, was given for 18 days and then reduced to 50 mg. 3 times weekly. The serum calcium decreased to 11.2 mg. per cent, and the serum phosphorus fell to 2.6 mg. per cent. Within 1 month there was relief of pain, and the patient remained comparatively asymptomatic until the 82d day of therapy, when nausea and diplopia occurred. The therapy was omitted, and the nausea disappeared within 2 days. Testosterone was resumed after a 5-day interval. Four weeks later (120 days after the initiation of testosterone therapy), because of drowsiness and increasing pain, the patient was admitted to the Pondville Hospital with suspected hypercalcemia. Chemical studies on admission were serum calcium, 13.4 mg. per cent; serum phosphorus, 3.7 mg. per cent; NPN, 39.0 mg. per cent. Since these values were similar to the initial studies, hormone therapy was continued. Suddenly, 9 days later, the patient became very drowsy and nauseated. The serum calcium was 19.0 mg. per cent; serum phosphorus, 3.6 mg. per cent; and NPN, 57 mg. per cent (Chart 7). The testosterone was omitted immediately, and 250 cc. of 2.5 per cent sodium citrate was given intravenously by slow drip over a 4-hour period. By the next day the nausea and vomiting had disappeared; the serum calcium was 17.7 mg. per cent and the NPN 70 mg. per cent. During the ensuing 25 days the serum calcium and NPN gradually decreased to normal. Three months later the patient was ambulatory and nearly free of pain without medication or hormone therapy. However, the disease slowly progressed, and the patient died 6 months later.

Comment.—This case again demonstrates a delayed hypercalcemia during androgen therapy. In this instance, however, the more profound chemical changes appearing after clinical recognition of the syndrome were undoubtedly due to continua-

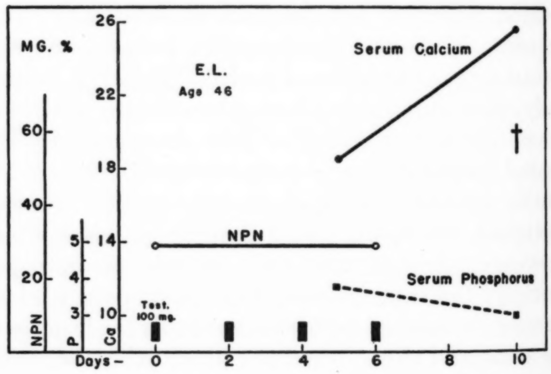

Chart 8.—Case VII: Illustration of a rapid onset of hypercalcemia. A serum calcium of 25.7 mg. per cent was recorded.

tion of the therapy. Sodium citrate infusion apparently combatted the complication rapidly, thus hastening recovery and protection of the patient. However, cessation of therapy alone might have accomplished the same result.

Case VII

E. L., age 46. P.H. #27023 (Chart 8). This patient was admitted to the Pondville Hospital in November, 1948, 6 months after a radical mastectomy, because of extensive, progressing, cutaneous, pulmonary, and osseous metastases. There were no symptoms of spontaneous hypercalcemia. Testosterone propionate, 100 mg. intramuscularly every other day, was commenced on December 4, 1948. Blood chemical studies were in-

advertently omitted on admission, but 5 days later the serum calcium was 18.5 mg. per cent; serum phosphorus, 3.8 mg. per cent; NPN, 29 mg. per cent; and the total protein, 6.8 gm. per cent. Consequently, testosterone was omitted when these chemical findings were available. The patient was asymptomatic until the onset of nausea on the 8th day. The following day the patient was disoriented, comatose, and incontinent of urine and feces. The patient died on the 10th day when the serum calcium had risen to 25.7 mg. per cent and the serum phosphorus had decreased to 2.0 mg. per cent (Chart 8).

Comment.—This case illustrates the rapidity with which hypercalcemia may progress after testosterone therapy. Even though preliminary studies were omitted and spontaneous hypercalcemia may have been present, clinical manifestations did not appear until 8 days after initiation of therapy and 3 days after elevation of the serum calcium was demonstrated. The serum calcium levels in this patient were the highest recorded in our series. It is possible that with prompt recognition and therapeutic intervention with sodium citrate infusions and other measures, the end-result might have been delayed.

CASE VIII

A. R., age 55. M.G.H. #293271 (Chart 9). This patient was initially seen in September, 1944, with extensive cutaneous manifestations of recurrent breast cancer. X-ray therapy was given, followed by satisfactory responses, but local reactivaton appeared February, 1947. Consequently, therapy with diethylstilbestrol was instituted and continued from February to September, 1947, with excellent regression of the disease. The process remained quiet until July, 1948, when severe pain developed due to widespread osteolytic metastases forcing the patient to bed. The primary focus was inactive. Testosterone propionate, intramuscularly, was commenced at a dose level of 50 mg. 3 times weekly. The serum calcium and phosphorus were normal during the early course of therapy. An increased feeling of well-being and a decrease in pain were noted by the 3d month; but despite this the patient remained bedridden, and x-rays revealed progression of the disease. Two months later recurrent pain, nausea, and intermittent vomiting occurred. The serum calcium had gradually increased to 11.0 mg. per cent. During the 7th month of androgen therapy, new skin lesions were noted, nausea persisted, and the serum calcium had risen to 14.4 mg. per cent. Androgen therapy was discontinued 10 days later because of increasing symptoms and progression of the disease. Death occurred 10 days thereafter.

Comment.—In retrospect, it was noted that the serum calcium increased slowly during the period of androgen therapy. Immobilization of the patient and constant progression of the disease may have been the sole causes of the calcium rise. However, testosterone may have contributed to or precipitated the hypercalcemia. This case demonstrates that the symptoms of hypercalcemia may be subtle whether they occur spontaneously from advancing disease, metabolic shifts, or are induced by steroid therapy.

CASE IX

L. W., age 53. P.H. #23280. Three years after a radical mastectomy this woman became bedridden because of severe pain and debility from osseous metastases. Testosterone propionate, 50 mg., 3 times weekly, intramuscularly, was started on October 7, 1948. There was complete relief of pain, increase in strength, and mobilization of the patient within 6 weeks. However, at the end of the 8th week, severe pain recurred, and the patient was again confined to bed. Because of increasing drowsiness, nausea, and vomiting suggestive of hypercalcemia, this woman was admitted to the Pondville Hospital during the 11th week of treatment. The serum calcium was 15.1 mg. per cent; serum phosphorus, 3.3 mg. per cent; NPN, 43 mg. per cent; and the total protein, 7.0 gm. per cent. Three days later testosterone was omitted. After 7 days, the drowsiness had vanished, and the serum calcium had returned to normal. Other blood chemical measurements were likewise within normal range. The disease progressed, however, and death occurred 1 month later.

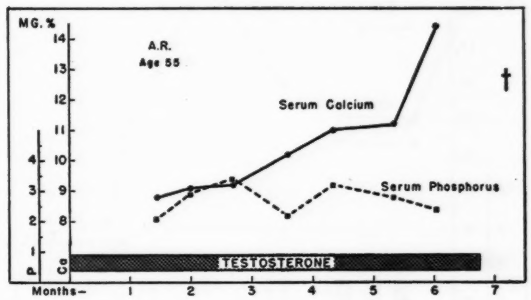

CHART 9.—Case VIII: Insidious development of hypercalcemia after several months of testosterone therapy.

Comment.—Though the patient was initially subjectively improved, hypercalcemia developed after 10 weeks of testosterone therapy. Discontinuance of therapy was followed by disappearance of the hypercalcemia but progression of the disease. Although difficult to evaluate, it is con-

ceivable that the hypercalcemic state may have accelerated the later course of the disease.

Case X

E. B., age 62. M.G.H. #609586 (Chart 10). Seventeen months after a right radical mastectomy, this woman developed osseous metastases in the pelvis. Pain in the leg was relieved by irradiation therapy. Three months later, extensive osseous and pulmonary metastases were demonstrated. Unfortunately, no preliminary chemical determinations were obtained.

The patient was given three injections of 50 mg. each of testosterone propionate, intramuscularly, on alternating days, beginning May 3, 1951. In conjunction with this, diethylstilbestrol was given for 5 consecutive days in daily doses of 10, 15, 15, 10, and 5 mg. On the 3d day of therapy, nausea,

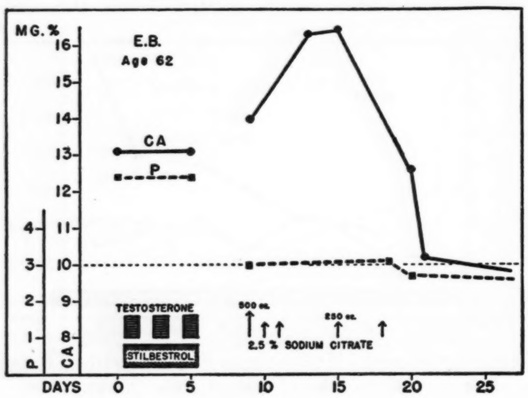

Chart 10.—Case X: Hypercalcemia induced by combined androgen and estrogen hormone therapy.

belching, and epigastric distress occurred. The nausea increased in severity and persisted during the subsequent 6 days. Hormone therapy was discontinued the 5th day, and intravenous infusions were begun.

Drowsiness, which was noticed the 6th day, increased until the 9th day, at which time the patient was difficult to arouse, unco-operative, and incontinent. The serum calcium was 14.0 mg. per cent; serum phosphorus, 3.0 mg. per cent; NPN, 45 mg. per cent; serum sodium, 121.4 meq/liter; serum chloride, 76 meq/liter; serum potassium, 3.1 meq/liter; and CO_2, 39.2 meq/liter.

Because of the obvious hypercalcemia and moderate dehydration, further infusions were given. In addition, 500 cc. of 2.5 per cent sodium citrate were administered intravenously the 9th day, followed by 250 cc. on each of the 2 following days. Nausea disappeared, and the patient became more alert. The 12th day the patient appeared icteric. The serum calcium the 13th day was 16.3 mg. per cent, and the total van den Berg 1.8 (Chart 10). The Sulkowich test for urinary calcium was 3+. On the 14th day the patient was mentally alert, and the mental confusion disappeared gradually thereafter. Because the serum calcium was still 16.4 mg. per cent on the 15th day, 250 cc. of 2.5 per cent sodium citrate was again administered and the dose repeated 3 days later.

The 21st day the serum calcium was 10.2 mg. per cent, and other serum chemistries were normal. The urinary calcium was 1+. On the 44th day the serum calcium was 8.8 mg. per cent; serum phosphorus, 2.4 mg. per cent; and NPN, 21 mg. per cent. No further hormone therapy was given. Despite x-ray irradiation, there was progression of the disease during the ensuing 6 months.

Comment.—This illustrates again the rapidity of onset of hypercalcemia that may occur with even small doses of hormone and the necessity to recognize the condition early and to combat dehydration. Since this was the third case in which bilirubinemia occurred without known liver disease, this may well be a characteristic of the hypercalcemia syndrome.

Case XI

L. T., age 54. M.G.H. #734994 (Chart 11). Two months following a right radical mastectomy for carcinoma of the breast, this woman complained of acute low back pain. Roentgenological examination revealed a metastatic lesion in the eleventh thoracic vertebra. The alkaline phosphatase was 3.5 units (Bodansky) per cent and the NPN, 35 mg. per cent.

Testololactone, 50 mg. 3 times a week, intramuscularly, was begun on July 31, 1951. One week later the serum calcium was 7.1 mg. per cent; serum phosphorus, 2.9 mg. per cent; and alkaline phosphatase, 3.9 units per cent. Because there was no relief of back pain after 2 months of therapy, testololactone was discontinued. Three weeks later the back pain increased, and a 2-cm. hard node could be palpated in the right supraclavicular area. The alkaline phosphatase was 13.7 units per cent. No serum calcium determination was done.

Because of the increase in back pain, testosterone propionate, 100 mg. 3 times a week, intramuscularly, was commenced on October 17, 1951. Diminution of back pain occurred the first 2 weeks of therapy. The 3d week a sudden increase in pain forced the patient to bed and was followed by anorexia and drowsiness. On admission to the hospital, 3 weeks after testosterone therapy was begun and 2 days after the last injection, the patient was nauseated, lethargic, and sweating profusely. The supraclavicular node was un-

changed. Roentgenological examination revealed diffuse osteolytic lesions in the spine and pelvis. The vertebral lesion in T-11, however, appeared dense. The serum calcium was 13.0 mg. per cent; serum phosphorus, 4.3 mg. per cent; alkaline phosphatase, 16.3 units per cent; total protein, 6.3 gm. per cent; and NPN, 100 mg. per cent (Chart 11). A Sulkowich test for urinary calcium was 3+. Analysis of spinal fluid revealed a calcium of 5.6 mg. per cent; phosphorus, 1.4 mg. per cent; and total protein, 32 mg. per cent. The serum electrolytes and van den Berg were normal.

Intravenous fluids were administered daily for 4 days. On the 2d hospital day, 250 cc. of 2.5 per cent sodium citrate were given intravenously. On the 4th hospital day, the patient was alert and no longer nauseated. The serum calcium was 11.2 mg. per cent; the urinary calcium excretion, 238 mg. in 24 hours; and a PSP excretion test was 35 per cent in 2 hours. The 10th hospital day the serum calcium was 9.5 mg. per cent; serum phosphorus, 3.2 mg. per cent; and the NPN, 38 mg. per cent. Spinal fluid calcium was 4.5 mg. per cent; phosphorus, 1.7 mg. per cent; and total protein, 40 mg. per cent.

Because of the widespread metastases and persistent back pain, and in an attempt to study further this syndrome of hypercalcemia, testosterone propionate, 100 mg. intramuscularly on alternate days, was begun the 13th hospital day. The serum calcium was 8.9 mg. per cent; alkaline phosphatase, 27.2 units per cent; and urinary calcium excretion, 46 mg. in 24 hours (calcium intake was 250 mg. daily). No change in the serum calcium or phosphorus followed. The alkaline phosphatase rose steadily to 71.2 units per cent by the 23d hospital day (Chart 11). The liver could not be palpated, nor was jaundice observed. The NPN was 21 mg. per cent, and the PSP excretion 44 per cent in 2 hours. On the 29th hospital day the patient was discharged and androgen therapy continued. The alkaline phosphatase had decreased to 38.8 units per cent. On x-ray examination no change in the osteolytic lesions was noted.

During the next 5 months back pain disappeared, and the patient felt well. Because of alcoholism the testosterone was discontinued May 5, 1952. The liver was palpable 3 cm. below the costal margin. Subsequently, the liver became further enlarged, jaundice appeared, and death occurred July 5, 1952. Post mortem examination revealed extensive metastases; the liver was almost replaced by disease.

Comment.—Hypercalcemia accompanied immobilization of the patient due to rapid progression of osseous lesions. After the hypercalcemia subsided, reinstitution of androgen therapy resulted in a striking reduction of urinary calcium and a temporary elevation of alkaline phosphatase to 71.2 units per cent.

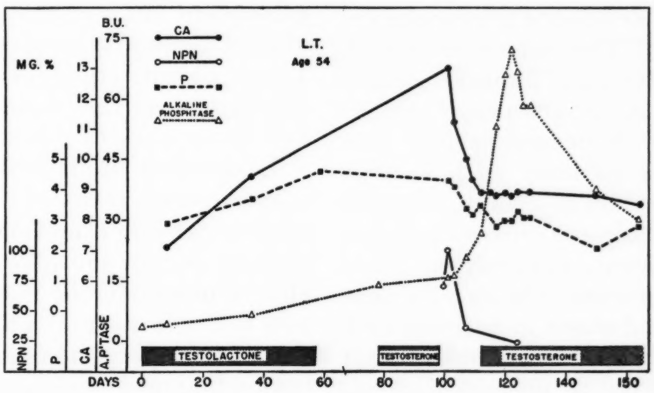

CHART 11.—Case XI: Hypercalcemia during testosterone therapy. A second course of therapy was accompanied by an increase of serum alkaline phosphatase to 71.0 B.U. per cent without evidence of liver disease.

DISCUSSION

Varying degrees of elevation of the serum and urinary calcium may occur in association with osteolytic metastases. These increases are found particularly in patients with breast cancer in which the metastases are predominantly osteolytic (11, 28). By contrast, the serum and urinary calcium are seldom increased in metastatic prostatic cancer where the osseous lesions are usually of the osteoblastic type, or in the rare breast cancer with osteoblastic metastases. When profound increases in calcium levels occur in the presence of osseous metastases, they are almost invariably accompanied by characteristic signs and symptoms. This extreme state may be designated as the "spontaneous hypercalcemic syndrome." Intermediate variations in the chemical and clinical

findings are more common. Under these circumstances, the symptoms and signs are usually subtle or absent. The diagnosis as a rule is made only by laboratory studies. Thus, it is often difficult to detect clinically the hypercalcemic state in its early stages, though it occurs spontaneously in 12–14 per cent of untreated patients with osseous metastases from breast cancer (34).[3]

It appears from the present study and other reports that an "induced hypercalcemic syndrome" may occur in association with steroid hormone therapy for osseous metastases from breast cancer (1, 19, 28, 34). Clinically, this is characterized by apathy, anorexia, nausea, vomiting, and drowsiness. In severe hypercalcemia weight loss, dehydration, disorientation, stupor, coma, vascular collapse, and death may occur.

In our experience, no cases clinically free of skeletal metastases have developed hypercalcemia during hormonal therapy. This is consistent with other reports and indicates that "induced" hypercalcemia does not occur in the absence of osseous involvement. Estrogenic hormones seldom produced hypercalcemia in patients with osseous metastatic lesions from breast cancer, whereas the occurrence of this syndrome during androgen therapy, although infrequent, was more common. With the use of either hormone, the development of the symptoms described above in patients with demonstrable osseous metastases from breast cancer should be considered as presumptive evidence of an impending hypercalcemia, even though the laboratory findings are within normal limits. Absence of x-ray evidence of osseous metastases or atypical laboratory data, however, does not completely exclude the possibility of hypercalcemia, since the defects are not always detectable. If hypercalcemia should occur during hormone administration without clinical evidence of osseous disease, the question of such involvement should be raised. Similarly, we consider an elevation of the serum alkaline phosphatase during hormone therapy in the absence of detectable liver disease presumptive evidence of osseous involvement. This indicates increased osteoblastic activity (bone repair) and may be of diagnostic significance.

Anorexia, nausea, vomiting, and apathy are fairly common early symptoms in patients with nonmalignant diseases treated with large doses of estrogens and are occasionally seen with androgen therapy (21). This is even true when an osseous disease such as osteoporosis is the primary reason for the treatment. There is no convincing evidence to indicate that the symptoms are due to hypercalcemia so that they must be attributed to some other action of the hormones. The mechanism is not known, but it has been demonstrated that steroid hormones, when used in sufficient amounts, are anesthetic in animals, which would help explain the drowsiness (33). In any event, the nausea, vomiting, diarrhea, and apathy that occur during hormone therapy of breast cancer in the absence of x-ray or laboratory evidence of osseous metastases may be due to hormone actions other than those causing hypercalcemia.

In the present series of eleven patients with advanced breast cancer, hypercalcemia occurred early in the course of hormone therapy in four. Five patients developed hypercalcemia at later periods during androgen therapy. Two patients who had an initial favorable response with testosterone therapy rapidly developed the syndrome during subsequent trials of the hormone for reactivation of the disease after rest periods (Cases IV and V). Three patients apparently died because of hypercalcemia, seven died from progression of the cancer and one is alive (Table 1).

Laboratory studies of the "induced" hypercalcemic state revealed an increase in serum and urinary calcium excretion and a negative calcium balance. Serum phosphorus levels were more variable. During steroid hormone administration in two patients without obvious renal impairment, the serum phosphorus decreased with a corresponding increase in urinary phosphorus. In one of these patients (Case I) the sequence of events was similar to that following the administration of parathyroid hormone in that an initial increase in urinary phosphorus was followed by a fall in serum phosphorus, a rise in serum calcium and hypercalcuria. In four patients with renal damage, as determined by an elevated NPN and impaired PSP excretion, the serum phosphorus was elevated. In five patients without evidence of renal disease the serum phosphorus was normal (Table 1).

The excretion of excessive amounts of calcium by the kidneys may be followed by calcinosis of renal tubules and deposition of calcium in glomerular tufts with resulting progressive renal damage (11, 12, 19). This impairment of renal function may become so marked that azotemia follows. Hypercalcemia and hypercalcuria, apparently induced by steroid hormone therapy, were accompanied by induced renal insufficiency in four patients, as judged by elevated serum NPN, impaired PSP excretion, and other routine tests. In one of these women (Case V), permanent renal damage resulted. Post mortem examination of the kidneys of another patient (Case IV) with

[3] I. T. Nathanson and B. J. Kennedy, unpublished data.

renal insufficiency demonstrated scattered calcium deposits (12). Based on the usual criteria, six patients suffered no renal damage during hypercalcemia and the renal status of all the patients was apparently normal initially. It should be stressed, however, that other and more specific tests of renal function were not routinely performed. Hence, subtle abnormalities may not have been detected.

Elevation of the serum bilirubin occurred in three cases (Cases I, III, and X) during the period of hypercalcemia. This might possibly be explained by red blood cell hemolysis or by increased viscosity of the blood (7, 25). There was no evidence of hepatic failure to account for the bilirubinemia.

Since the serum total protein was normal in all cases, the elevation of serum calcium would appear to be due to an increase in diffusible calcium, unbound to protein, and therefore capable of passing through tissue membranes.

The constancy of cerebrospinal fluid calcium is well known. The cerebrospinal fluid calcium is apparently in ionized form and identical with the diffusible fraction of serum calcium. The usual association between values for cerebrospinal fluid calcium and diffusible serum calcium is lost under conditions of abnormal serum calcium concentrations (25). In Case XI, the cerebrospinal fluid calcium was 5.6 mg. per cent (normal, 4.5–5.6 mg. per cent) when the serum calcium was 13.0 mg. per cent and 4.5 mg. per cent when the serum calcium was 9.3 mg. per cent. In this case the calcium of the cerebrospinal fluid paralleled the serum calcium but still remained within normal limits.

The following speculations have been considered in an attempt to explain the mechanism of action of steroid hormones in the production of hypercalcemia in patients with advanced breast cancer and osseous metastases:

1. Barriers may be removed that permit the metastatic tumor to grow unhampered, to be followed by marked demineralization of bone which results in an increase of serum and urinary calcium excretion (26–28).

2. The hormone may produce acceleration of tumor growth, resulting in more rapid destruction of bone and flooding of the blood stream with calcium (13).

3. Immobilization of an active individual may lead to continued bone absorption at a normal rate, decrease of bone formation, hypercalcemia, hypercalcuria, and even renal impairment. This has been observed particularly in children during periods of sudden immobilization of the extremities due to fractures or paralysis and in immobilized patients with osteitis deformans (5, 6). The degree of hypercalcemia depends on the discrepancy between demineralization and bone formation.

Immobilization may be involved in the hypercalcemia and hypercalcuria of patients with breast cancer treated with hormones. Six of these patients were already bedridden. Three were less active than normally, but were not confined to bed (Cases I, IX, and XI). Hypercalcemia developed in two ambulatory patients (Cases III

TABLE 1

ALTERATION FROM NORMAL OF THE CALCIUM, PHOSPHORUS, AND NONPROTEIN NITROGEN LEVELS DURING HYPERCALCEMIA

Case		Hormone	Serum			Urine		Cause of death
			Calcium	Phosphorus	NPN	Calcium	Phosphorus	
I	L.B.	Stilbestrol	++		0	++	++	Progression of disease
II	M.T.	Testosterone Stilbestrol	++	0	0			Progression
III	J.W.	Testosterone Stilbestrol	++		0	++	++	*Hypercalcemia*
IV	C.H.	Testosterone	++	++	++	++		Progression
V	A.F.	Testosterone	++	++	++	++		Progression
VI	D.F.	Testosterone	++	++	++			Progression
VII	E.L.	Testosterone	++	0	0			*Hypercalcemia*
VIII	A.R.	Testosterone	++	0				*Hypercalcemia*
IX	L.W.	Testosterone	++	0	0			Progression
X	E.B.	Stilbestrol Testosterone	++	0	0	++		Alive, disease progressing
XI	L.T.	Testosterone	++	++	++	++		Progression

and IV), but the symptoms of the syndrome forced them to bed. Hence, immobilization does not appear to be the sole factor involved, but may contribute significantly to the alterations observed.

4. Prolonged ingestion of vitamin D may produce a syndrome of hypercalcemia and tissue calcification (10, 14). However, the sequence of events in vitamin D toxicity maintains a hypercalcemia for longer than that seen in hormone-treated patients with advanced breast cancer. None of these patients received supplementary vitamin D.

5. In one case (Case I) the metabolic effects during stilbestrol therapy closely resembled those produced by the administration of parathyroid extract, even to the sequence in which they occurred (4). However, the parathyroid glands of those patients who succumbed to hypercalcemia appeared normal at post mortem examination.

6. A "spontaneous hypercalcemia" may occur in patients with extensive osteolytic metastases from various neoplasms, particularly breast cancer (15). The "induced hypercalcemic syndrome" of hormone therapy does not appear to be solely accounted for by spontaneous occurrences, since it occurred during the initiation of hormone therapy and in other cases was reproducible on re-institution of hormone therapy. Furthermore, the rapidity of onset in some cases is in contrast to the slower development of spontaneous hypercalcemia.

Management of "induced hypercalcemia."—The occurrence of hypercalcemia as a complication of hormone therapy of breast cancer does not contra-indicate its general use. Nor does the presence of a spontaneous elevation of serum calcium preclude hormone therapy. It is unusual for such hypercalcemia to be made worse by hormone treatment, and a decrease in the serum calcium level after commencing therapy is usually noted.[4] Frequent serum calcium and phosphorus determinations and renal function studies prior to and during hormone therapy may detect early alterations before the clinical symptoms of induced hypercalcemia occur. Mobilization promotes or maintains the normal stress stimulus to osteoblastic activity.

Once hypercalcemia is manifest, discontinuance of the hormone may be followed by a decrease of the serum calcium and disappearance of azotemia. Most important is adequate parenteral fluid to prevent severe dehydration and measures to correct the electrolyte imbalances that occur. A low calcium diet aids the reduction of the serum calcium. A further measure is the use of sodium citrate to reduce temporarily the amount of ionized calcium in the blood by formation of a soluble, non-ionized calcium citrate complex (23).[5] For this purpose, 250 cc. of 2.5 per cent sodium citrate can be administered intravenously and repeated in 4–6 hours if improvement does not occur, or to the point of developing a Chvostek sign of impending tetany. Caution must be exercised in regard to the total amount of sodium citrate given in a day. In this regard, knowledge of the state of renal function is imperative, since, if it is impaired, an alkalosis may be produced. This in itself may be as serious as the hypercalcemia. In some cases the measures advocated may be of no avail. The recent use of chelating agents may prove important in controlling the high blood calcium acutely though this has not been adequately delineated clinically.[6, 7]

SUMMARY

1. Hypercalcemia is a potential serious complication during androgenic and estrogenic hormone therapy of patients with advanced breast cancer and obvious osseous metastases.

2. Eleven cases of hypercalcemia occurring during steroid hormone administration are presented.

3. Metabolic studies of patients with "induced hypercalcemia" demonstrated an elevated serum calcium, hypercalcuria, altered serum and urinary phosphorus levels and occasionally renal insufficiency.

4. Immobilization, possible acceleration of tumor growth, and removal of barriers to the spread of cancer cells may contribute to the development of the hypercalcemia. The resemblance to the metabolic effects of parathyroid hormone is described in one case.

5. The detection and management of hypercalcemia are an essential part of steroid hormone therapy of advanced breast cancer.

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The Genesis and Growth of Tumors

VI. Effects of Varying the Level of Minerals in the Diet*

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Investigations concerning the influence of nutrition on the genesis and growth of neoplasms have emphasized the significance of the level of caloric intake and the proportions of dietary fat, protein, and vitamins (13). Little attention has been given recently to the possible role of the salt content of the diet in modifying the rate of formation or growth of tumors. In the earlier literature there are occasional reports that certain inorganic ions might be of specific importance, but generally these dealt with marked deficiencies or toxic excesses. There are no definitive reports, however, dealing with the influence of variations in the proportion of dietary salts (as a group) within what might be considered physiological limits. The lack of such studies and also the desire to complete the broad outlines of an over-all program on nutrition in relation to neoplasia (7–10, 12) prompted the present experiments. The results indicate that varying the salt content of the diet from 2 to 8 per cent has no noteworthy effect upon the relative frequency or rate of formation of the spontaneous mammary carcinoma and carcinogen-induced skin tumors of the mouse.

METHODS

The mice were of inbred strains, raised in our laboratory. At weaning they were divided into three groups, litter-mate distribution being employed as far as possible. They were housed five to a cage and were fed Purina Laboratory Chow Checkers until institution of the experimental diets.

In all experiments the mice received a partially purified diet which, excluding the salts, was composed of:

Casein, vitamin-free	22 per cent
Gelatin	2 "
Partially hydrogenated cottonseed oil	5 "
Cornstarch	71 "

In addition, the daily ration was supplemented with 0.02 ml. of yeast extract and the following amounts of crystalline B vitamins: thiamine, 28 μg; riboflavin, 8 μg; pyridoxine, 24 μg; pantothenate, 62 μg; niacin, 80 μg; choline, 2,000 μg. Ten U.S.P. units of Vitamin A, 1 U.S.P. unit of Vitamin D, and 0.4 mg. of Vitamin E were incorporated into the daily ration.

For the mineral component of the diet, Wesson's modification of the Osborne-Mendel salt mixture was utilized (14). It is a standard, adequate mixture made up as follows:

	Gm/kilo
$NaCl$	105
KCl	120
KH_2PO_4	310
$Ca_3(PO_4)_2$	149
$CaCO_3$	210
$MgSO_4$	90
$MnSO_4$	0.2
$FePO_4 \cdot 4H_2O$	14.7
$K_2Al_2(SO_4)_4 \cdot 24H_2O$	0.09
$CuSO_4 \cdot 5H_2O$	0.39
NaF	0.57
KI	0.05

This salt mixture was added to the basic ration at levels of 2, 4, or 8 per cent. Thus, each experiment included three groups of mice which were fed diets identical in composition and caloric content but differing in mineral content. The 2, 4, and 8 per cent levels were considered to represent low, moderate, and high proportions of dietary minerals, and, for simplicity, these terms will be used in referring to the rations and the respective experimental groups of mice.

In the first experiments with the spontaneous mammary carcinoma (Exp. 1) and the induced skin tumor (Exp. 3), the mice were fed ad libitum, and there were differences in food intake and body weights among the three groups of a study. Such differences were prevented, for all practical purposes, in Experiments 2 and 4, begun about 1 year later. This was accomplished by feeding the three groups of an experiment equicaloric amounts at slightly below ad libitum levels. Although there is relatively good agreement between the findings of the four experiments, those of the latter two are more specific.

The mice were allowed drinking water ad libitum. The routine execution of the experiments—preparation and storage of the diets, determination of food consumption and body weights, inspection for physical condition of the mice, gross

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recognition of tumors, and histologic examination of lesions—are described in previous publications (5, 10).

EXPERIMENTS AND RESULTS

GENESIS OF SPONTANEOUS MAMMARY CARCINOMA

Experiment 1.—Three groups, each consisting of 47 DBA female mice 21–26 weeks of age, were placed on partially purified diets which differed only in mineral content—2, 4, or 8 per cent. The diets were offered ad libitum. Within a few weeks the groups began to display differences in caloric intake that persisted throughout the experiment. The mean daily consumptions were 9.8, 9.4, and 8.9 Calories for the low-, moderate-, and high-salt groups, respectively. There were consonant differences in mean body weight (Table 1).

The study proceeded without untoward events and was terminated when the mice were 110 weeks old; at this time five, four, and three mice, respectively, were alive and without mammary carcinoma. The data on incidence of tumors and the average time at which they appeared are given in Table 1. It is obvious that there were no significant variations in the relative frequency and rate of formation of spontaneous mammary carcinoma between the groups receiving the diets containing 2, 4, or 8 per cent minerals. About one-fifth of the mice in each group developed multiple mammary tumors.

Experiment 2.—This study was patterned after Experiment 1, with the following pertinent differences: 65 DBA female mice, 10–13 weeks of age, constituted each group; *isocaloric food consumption was achieved by feeding the rations at the slightly restricted level of 9.0 Calories per day.* The control of food intake resulted in body weights of a similar order.

In each group five mice were alive and free of tumors at the termination of the experiment. Some particulars of the study and the results are listed in Table 1. Here, too, no noteworthy differences were observed between low-, moderate-, and high-salt groups as to the relative frequency and times of appearance of mammary carcinomas, nor was there an influence on the incidence of mice with more than one mammary tumor.

GENESIS OF SKIN TUMORS INDUCED BY CARCINOGENIC HYDROCARBONS

Experiment 3.—Three groups of 50 C3H male mice, 12–14 weeks of age, were placed on partially purified rations which differed only in mineral content—2, 4, or 8 per cent. The mice, fed ad libitum, consumed on the average 14.3, 13.9, and 13.4 Calories, respectively.

TABLE 1

INFLUENCE OF VARYING THE LEVEL OF DIETARY MINERALS ON THE GENESIS OF TUMORS

Experiment	Per cent minerals in diet	Mean daily caloric intake	Mean body weight (gm.) weeks of age 30	50	70	No.* mice	Per cent mice with tumors weeks of age 50	70	90	110	Mean time of tumor appearance (weeks)
1. Mammary carcinoma	2	9.8	27	30	30	42	10	41	57	71	72±3.1
	4	9.4	25	29	28	45	7	42	71	82	73±2.4
	8	8.9	25	25	24	40	5	22	52	75	80±2.8
2. Mammary carcinoma	2	9.0	28	31	27	56	5	32	55	80	79±2.8
	4	9.0	28	30	27	54	9	22	46	67	78±3.2
	8	9.0	27	28	26	52	4	38	53	73	73±2.4
			week of experiment† 0	20	40		week of experiment† 16	28	40	51	
3. Skin tumors	2	14.3	33	39	39	43	21	53	67	90	27±2.1
	4	13.9	33	37	36	48	8	31	60	69	29±1.8
	8	13.4	33	34	34	43	9	30	42	58	28±2.6
4. Skin tumors	2	11.2	35	35	35	63	6	51	89	95	30±1.4
	4	11.2	36	36	35	61	5	60	87	95	29±1.2
	8	11.2	34	34	35	61	3	59	83	89	28±1.3

* Number of mice adjusted for deaths of nontumor animals (1).
† Weeks after initial application of carcinogen.

Five weeks after institution of the experimental diets the animals received the first application of the carcinogen, a single drop of a 0.1 per cent acetone solution of 20-methylcholanthrene on the interscapular area. They were given a total of seventeen applications at twice-weekly intervals.

The experiment was ended 51 weeks after the first application of the carcinogen, at which time 0, 9, and 13 nontumorous mice were alive. The incidences of total skin tumors (papillomas and carcinomas) were 90, 69, and 58 per cent, respectively, for the groups given 2, 4, and 8 per cent minerals in the diet. The frequencies of carcinomas alone were in decreasing order also: 72, 56, and 44 per cent. Although the divergence in tumor in-

cidence is statistically significant, its biological pertinence must be evaluated in light of the concomitant decrease in food consumption and body weight values (Table 1). This is considered in the discussion.

Experiment 4.—The conditions were similar to those employed in Experiment 3, except for the following differences: 65 DBA male mice, 9–12 weeks of age, composed each group; *isocaloric food consumption was achieved by feeding the mice restricted rations containing only 11.2 Calories per day* (this resulted in comparable mean body weights); after the mice were on their experimental diets for 11 weeks, the applications of carcinogen were begun. Eighteen applications, each consisting of a single drop of a 0.3 per cent solution of 3,4-benzpyrene in acetone, were given at twice-weekly intervals.

The experiment was concluded 51 weeks after the first application of the carcinogen. The results are shown in Table 1. In contrast to the findings of Experiment 3, varying the mineral content of the diet from 2 to 4 to 8 per cent had no significant effect upon the incidence of total skin tumors or upon their average time of appearance. Not listed in the table are the percentages of mice with carcinoma; by the end of the experiment, 51 weeks, these were 56, 56, and 53, respectively, for the groups consuming 2, 4, and 8 per cent dietary minerals.

TABLE 2

THE RELATION OF DIETARY MINERALS TO THE INCIDENCE OF SPONTANEOUS HEPATOMAS

Per cent dietary minerals	Per cent mice with hepatomas	
	Exper. 3	Exper. 4
2	60	28
4	48	26
8	45	15

Genesis of Spontaneous Benign Hepatoma

Separate studies on the influence of the mineral content of the diet on the formation of spontaneous benign hepatomas were not conducted. However, inasmuch as C3H and DBA male mice on partially purified diets develop these neoplasms in appreciable frequency, the animals of Experiments 3 and 4 were routinely examined for hepatomas at autopsy. The incidences are shown in Table 2.

Although, in both studies, there was a decreasing incidence of neoplasms with the increasing proportion of dietary minerals, the differences are of small magnitude. It is concluded, tentatively, that varying the dietary salts from 2 to 8 per cent produces no important effect on the incidence of spontaneous benign hepatomas of the mouse.

Tumor Growth and Metastases

The actual rate of increase in the size of tumors was not determined. However, it was considered that an acceptable criterion of tumor growth is the survival time of the host—i.e., the interval between appearance of the neoplasm and death of the animal. Appropriate data have been gathered on spontaneous mammary carcinoma in C3H and DBA mice, indicating that the coefficient of linear correlation between the rate of tumor growth and the survival time of the host is approximately -0.5. In Experiment 1 the mean survival times were 8.9 weeks ± 0.65, 9.3 $\pm$ 0.74, and 9.3 $\pm$ 0.65 for the low, moderate, and high-salt groups, respectively; in Experiment 2 the values were 8.2 $\pm$ 0.78, 10.2 $\pm$ 0.78, and 10.9 $\pm$ 0.92. It is our opinion that alterations in dietary minerals, within the range utilized in these experiments, had no effect upon the growth of mammary neoplasms.

That the tumor-host relationship was not affected is also suggested by the data on grossly visible metastases to the lungs. In both experiments with mammary carcinoma, the relative frequency of metastases was approximately 10 per cent and did not vary with the salt content of the diet. In Experiment 4, the relative frequencies of metastases from skin carcinomas were 27, 25, and 26 per cent, respectively, for the low-, moderate-, and high-salt groups.

DISCUSSION

There is no reliable evidence that the genesis and growth of neoplasms are influenced significantly and specifically by altering the proportions of inorganic components natural to the diet. The literature contains many contradictory reports, most of which are based on studies that suffered from a variety of technical difficulties. Stern and Willheim reviewed the evidence and aptly concluded that there are few claims that have been verified without doubt (6). A principal error has been the failure to consider the role of the changes in nutritional state that usually accompany deficiencies or toxic excesses of dietary components. When such relationships were evaluated they generally sufficed to explain any observed effects on neoplasia. For example, injurious levels of fluoride definitely retarded the genesis of mammary carcinoma and lung tumors, but apparently no more than might be expected from the decrease in food intake and body weight (11). A diet deficient in potassium hindered the development of

tumor implants (2); again, the effect could be largely explained by the retarded growth of the deficient mice.

In the experiments reported in this publication, proportions of dietary minerals from 2 to 8 per cent were chosen, since it was reasoned that these levels approximate the lower and upper limits of the physiological range; furthermore, they are not trivial differences with respect to the animal. This is substantiated by studies on the influence of various salt levels on body and bone growth (3, 4) and by data of the present experiments. When fed ad libitum, the 8 per cent salt diet, as compared to the 2 per cent salt diet, resulted in suppression of food intake and body weight. In addition, the relative ad libitum water consumptions of the 2, 4, and 8 per cent salt groups were approximately 0.8:1.0:1.2.

The diets contained the minerals in Wesson's modification of Osborne-Mendel's salt mixture, as well as the inorganic ions present in the other dietary constituents. The salt mixture contains those trace inorganic elements for which need has been established; others not specifically incorporated into the mixture (cobalt and zinc) are probably present in trace amounts in both the salt mixture and in the other components of the diet. In the studies reported, no objective evidence of deficiencies was observed. At any rate, the findings and conclusions of this publication are related only to the stated conditions of the experiments.

It is likely that the results of Experiment 3 are valid—that there was a decreasing incidence of tumors with increasing proportions of dietary minerals. However, the results are in logical order and relation to the decreasing caloric consumption and body weight values, which in themselves may explain the findings (13). In fact, this interpretation seems so reasonable to us that the absence of a similar trend in Experiment 1 was unexpected.

With respect to the specific influence of the level of salt intake, however, principal attention must be given Experiments 2 and 4, in which food intakes were kept isocaloric, and resulting body weights were similar. In these studies no significant modification of the genesis of neoplasms occurred as a consequence of varying the mineral content of the diet from 2 to 4 to 8 per cent.

SUMMARY

The influence of varying the salt content of the diet on the genesis and growth of the spontaneous mammary carcinoma and carcinogen-induced skin tumors of the mouse was investigated. The studies utilized partially purified rations containing a standard salt mixture at levels of 2, 4, or 8 per cent of the diet—considered to be the physiological range for dietary minerals. When the experimental conditions were controlled so that the groups of mice were equivalent as to mean caloric intake and body weight, no significant differences were observed in the incidences or mean times of appearance of the neoplasms, or in their rate of growth.

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Studies on the Intracellular Composition of Livers from Rats Fed 2-Acetylaminofluorene*

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Price and his associates (17, 18, 19) found alterations in the amounts of protein, nucleic acids, and riboflavin in various fractions of the liver during the induction of liver tumors by a series of aminoazo dyes of widely different carcinogenic activities. The changes induced by the more active carcinogens made the composition of the liver approach that of the tumors induced by the parent compound, 4-dimethylaminoazobenzene (20).

Griffin *et al.* (6) determined the effect of 2-acetylaminofluorene (AAF) on the composition of rat liver during a prolonged period of feeding the compound. They found that the concentrations of total nitrogen, riboflavin, and pentosenucleic acid (PNA) decreased toward those found in the tumors induced by this carcinogen, while the concentration of desoxypentosenucleic acid (DNA) per gram of liver remained nearly normal.

In the present study, the intracellular changes in the content of nucleic acids, riboflavin, and protein-nitrogen were followed during hepatic carcinogenesis by AAF by analysis of the fractions obtained by differential centrifugation of liver homogenates.

METHODS

Male albino rats,[1] with an initial weight of 200–220 gm., were fed ad libitum a grain diet (12) containing 0.05 per cent of AAF for the first 4 weeks. To prevent an excessive mortality, the concentration of the carcinogen was reduced to 0.04 per cent for the remainder of the experiment. The rats were killed with ether, in representative groups of three or four, at 4, 7, 14, 25, and 27 weeks. Gross tumors were present in all the livers from the rats killed at 25 and 27 weeks, and the results of these analyses were averaged. The liver tissue analyzed at these times was carefully dissected away from the tumors, but the possible presence of a few microscopic tumors could not be excluded. Firm white tumors, less than 1 cm. in diameter and grossly free of necrosis, were used for fractionation. The tumors were obtained from other experiments in which the carcinogen was fed at a level of 0.036 per cent; tumors from six to twelve rats were pooled for each fractionation. Control analyses were made on the livers of four rats at the beginning of the experiment, and on two groups of four rats kept for 34 weeks on the same diet without the carcinogen.

All operations were carried out at 0–5° C. The livers were perfused via the vena cava with cold 0.14 M sodium chloride (23). Since liver tumors receive blood only via the hepatic arterial system (1), the tumors were not perfused under these conditions. Grossly, they did not appear to contain much blood, however. Tissue samples were fixed for microscopic examination, and then the livers or tumors were pooled, minced, and homogenized; an aliquot of the homogenate was separated into nuclear, large granule, small granule, and supernatant fluid fractions, as previously described (17, 18, 20). Each of the liver fractions and the original homogenate was analyzed for protein-nitrogen (25), nucleic acids[2] (22), and riboflavin (2).

The nuclei in two aliquots of the homogenate were counted (19), and from these counts the numbers of nuclei per gram and per whole liver were calculated. The analytical results were expressed as the amount in the average cell and in the average whole liver. The liver cells consisted mainly of the parenchymal cells and the newly-formed cells within the lobules, since the connective tissue and the blood vessels and bile ducts of the periportal spaces are largely removed by passage through a mincer (17).

Tissues were fixed for microscopic examination in Mossman's fixative (7) and in Regaud's fixative (3); the sections were 10 μ thick. The DNA content of individual nuclei was measured by a microspectrophotometric method (14) after Feulgen staining of the material fixed in Regaud's solution.[3] Nuclear diameters were measured on an arbitrary scale with an eye-piece micrometer and an oil immersion lens. Only those nuclei were measured which were entirely within the section or from which only a very thin slice had been removed; this was determined by careful focusing.

RESULTS

Although the rats gained an average of 13 gm. during the first 4 weeks, six of the 36 animals died between the 3d and 6th weeks. These deaths were apparently caused by the severe hepatic damage. The level of carcinogen was lowered from 0.05 to 0.04 per cent at 4 weeks, and all the rats alive at 6

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[1] Holtzman-Rolfsmeyer Rat Co., Madison, Wis.

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[2] The PNA analyses at 7 weeks were unreliable owing to technical difficulties and are not included.

[3] We are indebted to Dr. Hans Ris for the use of the microspectrophotometric equipment.

weeks survived until they were killed for analysis or developed tumors at 5 or 6 months.

During the first 4 weeks, the livers lost approximately one-third of their weight (Table 1). However, the number of cells per liver (as determined by the nuclear counts) was unchanged, so that the average cell decreased in weight from approximately 7 to 4.5×10^{-9} gm. The average cell weight remained at this low level for the remainder of the experiment; the cells of the tumors induced by AAF had an average weight of 2.5×10^{-9} gm.

Between the 4th and 7th weeks the liver cell population nearly doubled, and further rapid increases occurred throughout the remainder of the carcinogenic period. An accurate estimation of the total number of cells per liver was not possible at 25 and 27 weeks, when large necrotic tumors were present, but the total population (liver and liver tumor cells) was at least 8×10^9 cells.

The amounts of DNA, PNA, protein-nitrogen, and riboflavin in each fraction of the average liver cell for each of the times studied are given in Chart 1. The amount of DNA per cell remained relatively constant, and in all cases, including the fractionations of tumor, less than 10 per cent of the DNA was recovered in extranuclear fractions. In general, there was a marked decrease in the quantities of the other constituents in the various fractions during the first 4 weeks, and the minimum values were obtained at this time. The amounts of these constituents remained at their minimum levels in the nuclear fraction through the 14th week, but returned nearly to the initial level by the 25th week. In the large granule fraction, where the early drop was greatest, the amounts of these constituents remained at or near the minimum values throughout the experimental period. In the small

TABLE 1

WEIGHTS OF THE LIVERS AND OF AVERAGE LIVER AND TUMOR CELLS

Diet	Time (weeks)	Av. liver wt. (gm.)	Livers: Cells/gm liver $\times 10^{-6}$	Av. wt. liver cells (gm. $\times 10^9$)	No. of cells/liver $\times 10^{-9}$
Stock	0	13.7	148	6.8	2.06
Grain plus AAF	4	9.2	220	4.5	2.05
" " "	7	13.1	283	3.5	3.70
" " "	14	22.8	258	3.9	5.90
" " "	25	38.3*	255	3.9	>8.00*
" " "	27	37.1*	198	5.5	>8.00*
Grain	34	23.4	125	8.0	2.92
"	"	18.9	134	7.5	2.54

No. fractionation	Tumors: Cells/gm tumor $\times 10^{-6}$	Av. wt. tumor cells (gm. $\times 10^9$)
1	428	2.3
2	402	2.5
3	512	2.0
4	333	3.0

* Including liver tumors.

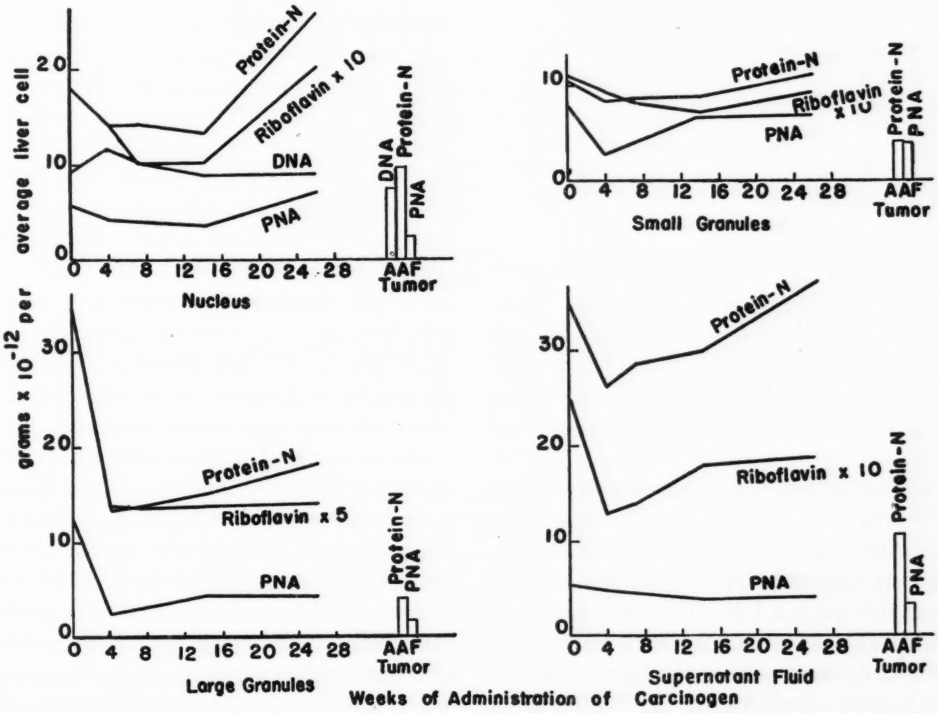

CHART 1.—Changes in cell constituents during carcinogenesis by AAF. The corresponding values for the diet controls killed at 34 weeks are given in Table 2.

granule and supernatant fluid fractions, the values generally began to approach the initial levels by the 7th or 14th week. The PNA content of the supernatant fluid of the average cell changed little throughout the experimental period. The analytical values for some of the liver cell fractions of rats maintained for 34 weeks on the same diet without carcinogen differed from those of the initial control animals, which had been maintained, prior to the experiment, on the stock colony diet of a commercial dog chow (Friskies, Carnation Co.) and the whole grain diet (12). All the protein values were higher in the diet controls than in the initial controls (Table 2); the PNA content of the large granules was lower, and that of the small granules higher, in the diet controls than in the initial control.

Since the decreases in the amounts of the large granules components were greater during the first 4 weeks than those of the other fractions and returned toward normal slowly (Chart 1), the intracellular distribution of the substances studied was quite different from normal throughout the period of administration of AAF. This altered intracellular distribution is similar to that of regenerating livers (16).

TABLE 2

AMOUNTS OF PNA AND PROTEIN-NITROGEN IN THE FRACTIONS OF LIVER CELLS FROM CONTROL RATS KILLED AT THE BEGINNING AND END OF THE EXPERIMENT

	PNA gm $\times 10^{-12}$/cell (weeks)		PROTEIN-NITROGEN gm $\times 10^{-12}$/cell (weeks)	
	0	34*	0	34*
Whole cell	322	292	1,030	1,330
Nucleus	55	52	180	266
Large granules	126	83	348	397
Small granules	78	107	106	181
Supernatant fluid	55	62	352	530

* Average of two fractionations.

The tumor cells contained much less protein and PNA in each fraction than normal liver cells (Chart 1). The tumors also contained much less riboflavin. In two cases the riboflavin content of the tumors was 1.0 and 1.1×10^{-14} gm/cell; the surrounding liver tissue contained 6.6 and 7.5×10^{-14} gm/cell. In most cases the quantities of protein, PNA, and riboflavin in the tumor cells were less than one-half the minimum values observed for the livers of rats fed the carcinogen, but the amount of DNA was essentially the same in the tumor cells as in the average liver cells from normal or AAF-fed rats.

The total quantities of protein-nitrogen, PNA, and riboflavin in each fraction from the average whole liver were also calculated. During the first 4 weeks of administration of AAF, the amounts of these constituents decreased in each of the fractions. After 4 weeks, however, marked increases in the amounts of each of these constituents were found; the increases were due largely to the increase in cell number. By 7 weeks the amounts of protein, PNA, and riboflavin in each fraction except the large granules exceeded the amounts in the corresponding fraction of normal whole livers. The amounts of these constituents in the large granules of the whole liver exceeded the normal levels by 14 weeks.

Microscopic observations.—At 4 weeks the parenchymal cells were uniformly smaller than normal, so that there were approximately 50 per cent more cells per microscopic field. This increase in cellular density corresponds to the 50 per cent increase in the number of nuclei found per gram of fresh liver. The cytoplasm of the cells in the central lobular region was coarsely clumped, forming large basophilic masses. The normal lobular architecture was preserved, however, and there was no indication of new cell types.

At 7 weeks the hepatic cells were even more shrunken than at 4 weeks, so that almost twice the normal number were present in each microscopic field. The peripheries of the lobules were partially overrun by cells of a new type, which gave the appearance of clusters of nuclei rather than cells. Although these cells resembled bile duct cells in some respects, they rarely formed ducts with lumens. At this time the cells with the coarse basophilic cytoplasm, which were seen only at the center of the lobule at 4 weeks, extended almost to the periphery, leaving only a rim of apparently normal cells. In a few areas the lobular architecture was replaced by well-demarcated nodules of hepatic cells. Necrosis was rare and confined to small areas.

At 14 weeks the parenchymal cells lay in dense sheets, and there was complete disruption of the normal lobular architecture. Many areas composed almost exclusively of bile ducts were seen, and these were usually associated with masses of dense, small, round or spindle-shaped cells and, occasionally, polymorphonuclear leukocytes. Areas of cells with coarse basophilic cytoplasm alternated with areas of cells containing a finely granular and diffusely basophilic cytoplasm, with no apparent reference to a lobular pattern. Of the cells with the coarse cytoplasm, a few contained a single large sharply defined vacuole. A marked variation in nuclear size was apparent at this time, and cells with giant nuclei seemed to be concen-

trated in the neighborhood of bile duct clusters. Numerous minute blood capillaries were found throughout the epithelial sheets, but few blood vessels with muscular walls were seen. The capillary lumina were occasionally distended to such a large size that they suggested sinuses.

At 25 and 27 weeks, little if any of the original architectural pattern remained. In some areas distension of the bile ducts had resulted in the production of small cysts. There were scattered foci of polymorphonuclear and small round-cell infiltration in the periportal spaces and throughout the parenchyma. A few small foci of necrosis were seen. The parenchymal cells were extremely variable in size, and a mosaic was formed of masses of large cells alternating with masses of small cells. The large cells had a homogeneous, relatively acidophilic cytoplasm and are apparently identical with those described by Cox *et al.* (4) in their pathological study of the lesions induced by AAF in the rat. The small cells had a coarse, granular basophilic cytoplasm and resembled those present at earlier times during the administration of the carcinogen. No tumor tissue was included in the sections.

The diameters of the parenchymal nuclei in the control livers varied considerably, and there was a tendency toward three peaks (Chart 2). As noted by previous workers (24), there was also a tendency for nuclei of different sizes to predominate in the periportal and centrilobular areas. Four weeks of exposure to the carcinogen produced no apparent change in the size distribution of the nuclei of the parenchymal cells. At 25 weeks discrete nodules were present. In each nodule there was little variation in the nuclear diameters of the cells, but the characteristic nuclear diameter varied from nodule to nodule (Chart 2).

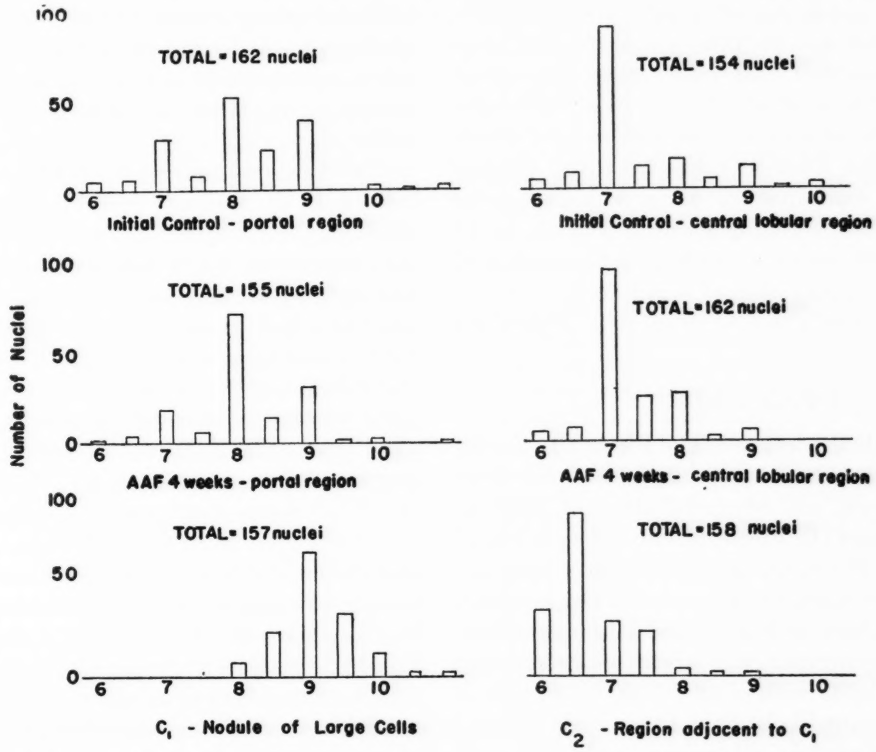

CHART 2.—Distribution of nuclear diameters in control livers and in livers from rats receiving AAF. The numbers along the abscissa are the nuclear diameters in arbitrary units, as measured with an eyepiece micrometer. The nodules C_1 and C_2 were found in the liver of a rat fed AAF for 25 weeks. Nuclei with diameters of 7 units have approximately one-half the volume of nuclei with diameters of 9 units.

Microspectrophotometric estimation of the DNA content of individual nuclei by the Feulgen reaction indicated that most of the large cells were polyploid. For this purpose, the Feulgen-reacting material per nucleus was expressed as $E \times D^2$, where E is the extinction coefficient ($\log I_0/I$) and D is the nuclear diameter in arbitrary units. Of 27 nuclei studied, twelve had diameters of 7.0 to 8.5 and relative absorptions of 134–195; these were apparently diploid resting nuclei. Seven nuclei with diameters of 10.0–11.3 had relative absorptions of 283–460 and were apparently polyploid

resting nuclei, while six nuclei with intermediate diameters of 8.3–9.5 and intermediate absorptions of 188–362 may have been diploid nuclei undergoing preparation for division. Two large nuclei with diameters of 10.0 and 11.3 were apparently diploid, since they had relatively low absorptions of 153 and 221, respectively; thus, the large size of these nuclei was not related to their content of DNA. The number of large nuclei in the livers from rats fed AAF for 25 weeks is relatively high, but it is not in proportion to the figures given, since large nuclei were purposely selected for analysis. The low incidence of giant diploid nuclei, about 8 per cent in a sample which favors large nuclei, suggests that the very numerous giant hepatic cells of Cox *et al.* (4) are polyploid cells.

No mitoses were seen on examination of about 2,000 cells in the liver sections from the control animals or on examination of an equal number from the rats fed AAF for 4 and 7 weeks. At 14 weeks the mitotic index was about 1 per cent, and at 25–27 weeks it was almost 2 per cent.

The tumors analyzed were of the hepatoma type.

DISCUSSION

A considerable depletion of the PNA, riboflavin, and protein content of each fraction of the liver cells resulted from the administration of AAF to rats. In most cases the minimum levels were found at 4 weeks, but the minima may actually have occurred somewhat earlier or later, since the analyses were made at rather widely spaced intervals. Similar decreases, expressed per gram of liver, have been observed by Griffin *et al.* (6) and Rutman *et al.* (21). It appears likely that these early alterations were produced in interphase cells, since no mitoses were seen in the sections taken at 4 weeks and the number of cells per liver (as determined by nuclear counts) was essentially the same as in the control livers analyzed at the beginning. Within the next few weeks there was an extensive proliferation of these altered cells, so that by 7 weeks there were nearly twice and by 14 weeks 3 times as many cells per liver as at the start of the experiment. These cells were, on the average, only one-half to two-thirds as large as normal liver cells. As a result, although the amounts of PNA and protein on a cellular basis were lower than in normal liver cells, the amounts per whole liver were actually equal to or greater than normal by 8–14 weeks. This observation is in essential agreement with that of Griffin *et al.* (6). The increase in cell number between 4 and 7 weeks apparently resulted from a relatively sudden burst of mitosis, since no mitotic figures were seen in the sections taken at either 4 or 7 weeks and since the distribution of nuclear size classes of the portal and centrilobular regions were so similar in the control and 4-week livers. At 14 and 27 weeks, a mitotic rate of 1–2 per cent was observed.

The apparent recovery of the cells at 25–27 weeks illustrates the difficulties inherent in interpreting data on the basis of the "average cell" after the cell population becomes diverse. At this time there were a considerable number of large cells which probably contained greater quantities of the various constituents than the small cells also present. However, when averaged together these widely different cell types gave values approximating those obtained with normal liver cells.

The tumors induced by AAF were composed of cells with an average weight approximately one-third that of normal liver cells. The tumor cells also contained much less protein, PNA, and riboflavin than normal liver cells and, in most cases, less than half as much as the liver cells from rats fed the carcinogen for 4 weeks. On the other hand, the DNA content of these cells was essentially the same as that of normal liver cells or of the damaged liver cells analyzed at any stage of AAF ingestion. When calculated on a fresh weight basis, the DNA content of tumors collected in four experiments ranged from 3.9 to 4.9 mg/gm, or approximately twice that of normal liver. This observation is at variance with the finding of Griffin and his associates (6) that the DNA content on a fresh weight basis was approximately the same for normal liver and for the tumors induced by AAF. The AAF-induced tumors vary from rather diffuse neoplastic areas to firm white nodules, and the latter type, diagnosed microscopically as hepatomas, were used in this study.

On the cellular level, at least, the results of the present study are in contrast to the conclusion of Griffin *et al.* (6) that the cellular alterations induced during carcinogenesis with AAF are essentially different from those induced by 3′-methyl-4-dimethylaminoazobenzene. Although the alterations produced in the liver by the ingestion of the two carcinogens appear quite different when expressed on a fresh weight basis, both compounds caused a considerable depletion of the protein, PNA, and riboflavin contents of most of the fractions of the average cell. The AAF data of Griffin *et al.* (6) have been recalculated with reference to DNA by Davidson and Leslie (5), who thus demonstrated early depletion of cellular constituents and later increases to or above the normal level.

With both carcinogens, the large granule fraction was the most severely altered (9, 18). As in the case of AAF, the ingestion of 3′-methyl-4-dimethylaminoazobenzene caused extensive depletion of the large granule fraction of the average cell prior to a sudden burst of mitosis at about 4 weeks (9). In both cases the tumor cells are smaller than normal liver cells and contain less of each component, except DNA, than the normal liver cells (5, 8, 9, 16).

As in the case of carcinogenesis by the azo dyes (18, 19), the average composition of the liver cells after 4 weeks' exposure to AAF approaches that of the tumor cells which arise at a later date. Only a few aberrant cell divisions might be required to convert some of these cells into cells with the composition of neoplastic cells (8). That cell divisions can occur in which the rate of accumulation of one cell constituent does not keep pace with the rate of cell division was shown with regenerating liver (16). Studies from this laboratory (10, 11, 13, 15) have suggested that the deletion of certain key proteins may be involved in the genesis of neoplastic growths. While data such as these show that a great decrease in the gross amount of protein per cell occurs during carcinogenesis (5, 9, 18, 19), the determination of the identities of such key proteins will depend on finer methods of analysis.

SUMMARY

1. Livers from rats fed 2-acetylaminofluorene for various times up to and including gross tumor development (4, 7, 14, 25, and 27 weeks) were homogenized and separated into nuclear, large granule, small granule, and supernatant fluid fractions, and each fraction was analyzed for protein nitrogen, nucleic acids, and riboflavin. The results were expressed in terms of the amount per average cell and per average liver. Livers from rats taken at the beginning of the experiment or maintained on the same diet without the carcinogen for 34 weeks and liver tumors induced by 2-acetylaminofluorene were analyzed in the same manner.

2. In general, the levels of protein, pentosenucleic acid, and riboflavin, when expressed on a cell basis, fell to minimum values at 4 weeks. These minimum values were either maintained or there were slow increases toward the levels found in normal liver cells. In general, the tumor cells contained about half as much of each constituent as was found in the liver cells after 4 weeks of treatment. The damaged liver cells and the tumor cells contained the same amount of desoxypentosenucleic acid as did normal liver cells.

3. At 4 weeks the number of cells per liver was essentially normal, but the cells were only about two-thirds as large as the usual liver cell. Thereafter, the cells retained their small size, except for some very large cells which arose late in the experiment, but the number of cells per liver was nearly twice the initial level at 7 weeks, and nearly 3 times the normal level at 14 weeks.

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Announcements

Recommendations Adopted by the Symposium on the Endemiology of Cancer of the Lung

held by

Council for the International Organizations of Medical Sciences under the auspices of World Health Organization

and

United Nations Educational, Scientific and Cultural Organization

Session held at

Institut du Cancer, Louvain, Belgium

July 21–24, 1952

SYMPOSIUM ON THE ENDEMIOLOGY OF CANCER OF THE LUNG

The Symposium on the Endemiology of Cancer of the Lung was convened by the Council for the International Organizations of Medical Sciences (C.I.O.M.S.), thus giving effect to a recommendation of the Committee on Geographical Pathology of Cancer under the International Cancer Research Commission.

The Symposium held its sessions at Institut du Cancer, Louvain, Belgium, from July 21 to 24, 1952, under the chairmanship of Dr. Harold L. Stewart. The scientific secretary during the preparatory stages of the symposium and its sessions was Dr. Johannes Clemmesen.

The following members were present.—

J. Maisin, M.D., President C.I.O.M.S., Professor of Pathology, University of Louvain.

H. L. Stewart, M.D., Chairman. Executive Secretary, Committee on Geographical Pathology of Cancer, International Cancer Research Commission. Chief, Laboratory of Pathology, National Cancer Institute, Bethesda 14, Maryland, U.S.A.

J. Clemmesen, M.D., Scientific Secretary, Associate Secretary, Committee on Geographical Pathology of Cancer, International Cancer Research Commission. Chief Pathologist, Director Cancerregisteret, Strandboulevard 49, Kobenhavn, Denmark.

K. Arnesen, M.D., Pathologist, University Institute for general and experimental Pathology, Oslo, Norway.

C. De Muylder, M.D., Agrégé de L'enseignement superieur, Université Catholique de Louvain. 497, Avenue Louise, Bruxelles, Belgique.

P. F. Denoix, M.D., Chirurgien des Hôpitaux de Paris, Chief de la Section du Cancer a l'Institut National d'Hygiène, 3, rue Léon Bonnat, Paris, France.

R. Doll, M.D., M.R.C.P. Statistical Research Unit, Medical Research Council, London School of Hygiene, Keppel Street, London W.C.I. England.

H. F. Dorn, Ph.D., Chief Office of Biometry, National Institutes of Health, Bethesda 14, Maryland.

Cuyler Hammond, Sc.D., Director, Statistical Research Section, American Cancer Society, 47 Beaver Street, New York 21, N.Y., U.S.A.

Sir E. Kennaway, F.R.S., M.D., Pathological Department, St. Bartholomew's Hospital, London E.C.I. England.

R. Korteweg, M.D., Victorieplein 45, Amsterdam, Netherlands.

J. Kretz, M.D., Cancer Specialist of the City of Wien, General Secretary of the Austrian Cancer Society, Universitatsstrasse 11, Wien I, Austria.

Morton Levin, M.D., Dr. P.H., Assistant Commissionar for Medical Services, New York State Department of Health, Albany, N.Y., U.S.A.

M. G. Neurodenburg, M.D., Medical Inspector of Public Health Service, Amsterdam, Netherlands.

A. Nielsen, Lic. act., The Danish Cancer Registry, Strandboulevard 49, Kobenhavn, Denmark.

E. Pedersen, B.M., Ch.B., Director, The Cancer Registry of Norway, Bestun, Oslo, Norway.

W. E. Smith, M.D., Assistant Professor, Industrial Medicine, New York University, 477 First Avenue, New York 16, N.Y., U.S.A.

P. E. Steiner, M.D., Professor of Pathology, The University of Chicago, Chicago 37, Ill., U.S.A.

R. E. Waller, B.Sc., Former Scientific Assistant, Pathological Department, St. Bartholomew's Hospital, London E.C.I., England.

Observers present.—

Dr. Robert A. Moore, Dean, School of Medicine, St. Louis 10, Missouri.

Dr. Marvin Kuschner, Consultant in Pathology, Institute of Industrial Medicine, New York University, 477 First Avenue, New York, N.Y., U.S.A.

Dr. R. Firket, Professeur, Institut d'Anatomie Pathologique Université de Liége, 2 rue des Bonnes Villes, Belgique.

Dr. Morgan, England.

Program

Dr. P. Steiner: Etiologic Implications of the Geographical Distribution of Lung Cancer.
Dr. J. Kretz: Cancer of the Lung in Austria.
Dr. Harold F. Dorn: The Incidence of Cancer of the Lung in U.S.A.
Dr. Ch. De Muylder: Cancer of the Lung in Belgium.
Dr. Neurdenburg: Report on Cancer of the Lung in Netherlands.
Dr. R. Korteweg: Report on Cancer of the Lung in Netherlands.
Dr. P. Denoix: Les Cancers Broncho-pulmonaires en France.
Dr. J. Clemmesen: Cancer of the Lung in Denmark.
Dr. M. Levin: Difficulties in Establishing Statistics on Cancer of the Lung.
Dr. R. Korteweg: The Application of a New Statistical Method in Cancer of the Lung.
Dr. Harold Stewart: Cancer of the Lung in Laboratory Animals.
Dr. William E. Smith: Occupational Factors in Cancer of the Respiratory Tract.
M. R. Waller and E. Kennaway: Studies on Cancer of the Lung.
Dr. Richard Doll: Studies on Cancer of the Lung and Smoking Tobacco in England.
Dr. K. Arnesen: Studies on Cancer of the Lung in Norway.
Dr. C. Hammond and D. Horn: Tobacco and Lung Cancer.

Committee Work and Reports

The purpose of the Symposium was to review our present knowledge on the Endemiology of Cancer of the Lung and to make precise proposals for the amplification of this knowledge.

The field of the endemiology of cancer will be broadly defined as comprising knowledge concerning variations in the distribution and behavior of cancer among various ethnological groups in different localities in relation to any relevant local factors. The first task of the Symposium therefore was to sift available information in order to determine what evidence there is as to the extent and character of the increase in number of cases and deaths ascribed to cancer of the lung, observed during the last decades mainly among males, in various countries and areas. However, the Symposium considered it equally important to study in detail the nature of the factors which may contribute to an increase in the number of cases of cancer of the lung and to review the possibilities for further studies of these factors. Finally, the Symposium found it desirable to propose uniform criteria for the acceptance and grouping of cases of cancer of the lung.

As a result of its studies the Symposium passed recommendations on the following subjects:

1. Criteria for the acceptance and grouping of cases of cancer of the lung for endemiological studies.
2. Publication of results of studies.
3. Review of available information on the endemiology of cancer of the lung, and of some possibilities for further studies.

Recommendations

Criteria for the acceptance and grouping of cases of cancer of the lung for endemiological studies:

The Symposium on the Endemiology of Cancer of the Lung, feeling that in order to establish valid statistics on cancer of the lung, there must be common agreement on the criteria used, stress the following preliminary, general considerations.

The term "Cancer" is synonymous with the term "Malignant Neoplasm" which includes carcinoma and sarcoma. The term "Carcinoma" is understood to be equivalent to the term "Epithelioma" as used in some countries to designate malignant epithelial tumors.

Cases of cancer of the larynx should not be classified under the same heading as cases of cancer of the lung. The general classification "Tumours of the respiratory tract" should be avoided.

Cases of secondary cancer of the lung must always be clearly distinguished from cases of primary cancer of the lung.

Histological examination constitutes the most valid proof of the existence of a cancer of the lung and should be made use of whenever possible.

The Symposium taking these general considerations into account adopted the following recommendation:

Recommendation 1.—The Symposium on the Endemiology of Cancer of the Lung recommends:

1.1.—That each case of cancer of the lung be classified anatomically in accordance with the "International Statistical Classification of Diseases, Injuries and Causes of Death" established in 1948, as revised in 1952.

The headings under which these cases may be listed are the following:

162.—Malignant neoplasm of trachea and of bronchus and lung specified as primary:

- −0 Trachea
- −1 Bronchus and Lung
- −2 Pleura

163.—Malignant neoplasm of lung and bronchus, unspecified as to whether primary or secondary.

165.—Malignant neoplasm of thoracic organs (secondary). In order to determine the anatomical site of cancer of the lung most accurately the following optional subheadings under item no. 162.1 are suggested:

A. Tumor located in bronchi, near the tracheal bifurcation.
B. Tumor located in bronchi, but directly or indirectly accessible to endoscopy.
C. Tumor of broncho-pulmonary location, not accessible to endoscopy.
D. Tumor of alveolar location.
For example, a carcinoma of the bronchus, located near the tracheobifurcation would be grouped as 162, 1.A.

1.2.—That in endemiological studies, whenever possible, the validity of the diagnosis of primary cancer of the lung should be classified according to the following system of priority based on the methods employed in establishing the diagnosis.

The following methods are available for establishing the diagnosis or primary cancer of the lung:

1. Case history and physical examination.
2. Radiological examination.
3. Bronchoscopic examination without biopsy.
4. Cytological examination of sputum and bronchial aspirates.
5. Cytological examination of pleural fluid.
6. Histological examination of metastases.
7. Histological examination of the primary tumor.
8. Necropsy examination.

The validity of diagnosis of primary cancer of the lung should be grouped following the order of priority given below:

First:

Positive result of histological examination of primary tumour of the lung, or
Positive result of histological examination of secondary tumor and evidence of primary tumor of the lung by radiological examination, bronchoscopy, or thoracotomy, or
Positive result of cytological examination and evidence of primary tumor of the lung by radiological examination, bronchoscopy, or thoracotomy, or
Autopsy.

Second:

Positive result of cytological examination only, or
Positive result of radiological examination only, or
Positive result of bronchoscopical examination only, or
Positive evidence at thoracotomy without biopsy.

Third:

Evidence based on case history and physical examination without further study, or
Death certificate as only evidence.

1.3.—That so far as possible, the various types of primary cancer of the lung shall be grouped according to the following histological classification:

a) epidermoid carcinoma (epidermoid epithelioma)
b) anaplastic carcinoma (anaplastic epithelioma; large, small and oat-cell carcinoma).
c) adenocarcinoma (glandular epithelioma).
d) mixed forms combining some or all of the preceding types of cells
e) bronchiolar carcinoma
f) malignant bronchial adenoma
g) malignant tumor of salivary gland type

This histological classification should be superseded by the "Manual of Tumour Nomenclature and Coding" whenever they are inconsistent in the hope that an international agreement will be reached on the proposed classification.

1.4.—That in order to implement the resolution on the classification of neoplasms according to histological type passed by the World Health Organization (WHO, techn. Rep. Ser., 1952, no. 53 p. 47).

1.4.1.—The American Cancer Society be invited to cooperate with the World Health Organization in the distribution of copies of the "Manual of Tumour Nomenclature and Coding" throughout the world for trial as recommended by the World Health Organiza tion.

1.4.2.—Lists of pathological terms actually used be drawn up in the various countries with the cooperation of World Health Organization and the International Cancer Research Commission for use in preparing an edition of the "Manual of Tumour Nomenclature and Coding" in order to meet the need of each country.

1.5.—That endeavor should be made to subdivide cases of primary cancer of the lung according to the degree of clinical extent or stage, following the general rules laid down for such staging by World Health Organization (loc. cit.).

Publication of results of studies.—The Symposium considered the methods available for statistical studies of geographical and other variations in the incidence of cancer and adopted the following recommendations:

Recommendation 2.—The Symposium on the Endemiology of Cancer of the Lung, considering that it is desirable to obtain a high degree of comparability of results of study in the field of endemiology of cancer, and being in full agreement on this point with the Symposium on Geographical Pathology and Demography of Cancer held at Regents Park College, Oxford, England, July 28 to August 4, 1950, and with the World Health Organizations Subcommittee on the Registration of Cases of Cancer (WHO, techn. Rep. Ser. 1952, 53), resolve to reaffirm the recommendation 2 adopted by the said symposium in Oxford, and thus recommends:

2.1.—That all authors and editors of future publications on the frequency of malignant disease shall provide information on the following essential points, if possible separately for each site affected:

2.1.1.—Total number of new cases in the area being studied.

2.1.2.—Distribution of the total population and of patients in the area with respect to race, sex, and age.

2.1.3.—Percentage of cases diagnosed in hospital.

2.1.4.—Percentage of cases diagnosed by histological examination.

2.1.5.—Percentage of cases verified by autopsy.

2.1.6.—Description of any subdivision undertaken (social, occupational, geographical, etc.)

2.1.7.—Statement on the type and location of hospitals from which statistics have been gathered.

(Considerations as given in *Rep. Symp. on Geographical Pathology and Demography of Cancer*, Oxford, 1950; sep. Vol. VII of *Acta*, Internat. Union against Cancer, 1951, and *Journal National Cancer Inst.*, 11:627, 1950.)

This recommendation was adopted as the result of the following considerations:

It was realized that the increasing efficiency in the therapy of cancer tends to make mortality statistics increasingly inadequate as a measure of the real incidence of malignant disease. Studies of the mortality from cancer may, however, still be useful for certain purposes such as studies of cancer of specific sites. It was especially pointed out that, if such studies are made for rural and urban populations, appropriate adjustments should be made for deaths of nonresidents.

It was agreed that, ideally, studies of cancer incidence in a given area should contain full information concerning the total number of new cases of malignant disease, diagnosed within the period under consideration as far as possible for each site separately. If special studies are carried out for a single site, information as to the total number of all sites within the material treated should be given whenever possible.

In addition to full subdivision with respect to primary site, a complete description should be given of the age, preferably in quinquennial groups, sex, and racial composition of the general population from which the cancer cases have arisen.

Cases with a diagnosis of "possible" or "probable" cancer may be shown by a total for each site, but should not be included in the statistical treatment. Although diagnoses of malignancy confirmed by histological examinations (biopsy or autopsy) are the most reliable, statistical data for such cases alone usually will be unrepresentative of all cases of cancer developing in a population. Special analysis of histologically confirmed cases may be useful in evaluating the reliability of the conclusions drawn from the total data, although the value of this test will vary with the percentage of cases verified histologically. Whenever possible, the percentage of cases histologically confirmed should be stated for each site separately.

When studies are made of the occurrence of cancer in various social classes, or other groupings, the criteria of such subdivisions should be clearly stated.

Studies of cancer incidence in urban and rural areas should give the basis for this classification as well as information concerning such factors as density of population, character of industries in the area, the type of cultivation, etc.

When conclusions are drawn from hospital cases alone full information should be given concerning the extent of the area served and the type of cases admitted to the hospital.

Publications should contain tables giving the fundamental figures mentioned above for the entire population as well as for the cancer patients, so that other workers will be able to make comparisons or analyses apart from those in the original publication. Likewise, all formulas used in the computation of results should be given in full so that readers can study both methods and results obtained.

It is desirable also that results should be illustrated by diagrams or graphs, so that readers specialized in other branches of cancer research may form an opinion of the results obtained without a detailed study of tables and figures. The omission of these details in order to reduce the expense of printing will decrease the value of the paper concerned.

Etiological Studies on Cancer of the Lung in Past and Future

The Symposium reviewed information available on the Endemiology of Cancer of the Lung, and arrived at the following conclusions:

Progressive increase in number of cases and deaths ascribed to cancer of the lung has been observed for several decades, mainly among males in a number of countries and areas. However, it seems equally clear that there has been—and still is—considerable controversy as to whether this increase is real in nature or only apparent, i.e., due to progress in medical science and improvement in diagnosis and reporting.

The Symposium believes that a significant part of this increase is absolute and represents a real increase in the number of people suffering from cancer of the lung. Further research as to the extent of this increase, or where it is not found, the conditions of its absence, should be undertaken.

The Symposium considered several possibilities with regard to etiological factors:

The smoking of tobacco—especially cigarettes—has often been regarded as a causal factor of cancer of the lung. While it would be impossible to accept tobacco smoking as the only cause of cancer of the lung, there is now evidence of an association between cigarette smoking and cancer of the lung, and that this association is in general proportional to the total consumption. Further research on this subject is imperative.

An extensive chemical analysis of the products of all forms of tobacco smoking with a view to establishing whether or not any known carcinogens such as 3,4-benzpyrene are present seems equally called for. Fluorescence spectroscopy offers a sensitive means for detecting small quantities of some polycyclic hydrocarbons under favorable conditions; the methods based on absorption spectroscopy are considered to be more reliable.

Atmospheric pollution is most likely to derive from effluvia and smoke from factories and domestic fires and by exhaust fumes from petrol and Diesel engines.

For these factors it is primarily desirable to establish definitely whether or not any relationship exists with the occurrence of the disease in man. This might be approached by statistical inquiries in which populations subject to different environments are defined and are observed over a period to determine their death rates from carcinoma of the lung, coupled with estimations of concentrations and analyses of the several kinds of air pollution in the relevant areas. In view of the considerable internal migration which takes place in some large countries, such investigations may be more feasible in, for example, the Scandinavian countries. Attention should be directed in the first instance to gross differences in pollution between different areas followed by more detailed analyses for possible carcinogens, such as 3,4-benzpyrene. Determination of carbon in lung post mortem offers a useful means of evaluating the extent of smoke particle retention.

Occupational hazards giving rise to lung carcinoma have been demonstrated in a number of industries, in particular in the handling of asbestos and chromates, in gas-workers, in a factory refining nickel and in certain mines bearing radioactive ores. New processes may not be proved to be free of risk of cancer for many years, and other occupational hazards may be detected in the future. Nevertheless, occupational hazards cannot be considered to contribute more than a small proportion of all cases of carcinoma of the lung. The important

causes must be sought in such aspects of the environment as are common to the majority of the population in countries in which a high incidence of the disease has been established.

Environmental factors other than those mentioned, but as yet unknown, may, however, enter the human body through ingestion, skin contact, etc. to exert a carcinogenic influence on the lung, although in so far as is known, the entry of environmental carcinogens into the human lung is by inhalation. Lung tumors induced in animals by materials introduced by other routes do not appear to have counterparts in human experience, but such possibilities must be borne in mind. Experimental studies should be conducted with special reference to inhalation technics and to sites of deposition of inhaled particles in the respiratory tract; also the susceptibility of a variety of species of animals should be tested. Certain simpler procedures may be useful for preliminary investigations of suspect substances. These simpler procedures include (1) intratracheal injections, (2) skin painting, (3) subcutaneous injection, (4) the tissue transplant technic. Experiments may also be conducted to examine the effect of (5) material deposited in the nose and oropharynx. It must, however, be remembered that it is man with whom we are ultimately concerned and that animal experiments should be interpreted in the light of observations made on the occurrence of the disease in man. This need not preclude the taking of immediate precautions against hazards to man suggested by animal tests.

Variations in susceptibility of the individual exposed to the influence of environmental factors should not be ignored and would seem to deserve further investigation; for example, it is not known to what extent sex differences in incidence of cancer of the lung can be explained by differences between the two sexes with regard to smoking habits.

Co-action of etiologic factors should also be taken into consideration with a view to the possibility that inhaled substances may have a cocarcinogenic influence or that conditions within the lung may have such an effect, which ultimately may influence a possible latent period for carcinoma of the lung.

Studies of the gross and histological types of tumors may be significant in the evaluation of results from other kinds of study, which should be carried out with full attention to the type of lesion. Thus, studies on histological changes in the respiratory tract, which may result from the influence of tobacco smoking, and similar studies on individuals exposed to industrial hazards, and not developing carcinoma of the lung may give significant results. In view of these considerations the Symposium adopted the following recommendations:

Recommendation 3.—The Symposium on the Endemiology of Cancer of the Lung recommends:

3.1.—That the Committee on Geographical Pathology of Cancer, under the International Cancer Research Commission, should stimulate the organization and execution of further studies on the following subjects with a view to the etiology and pathogenesis of primary cancer of the lung.

3.1.1.—The extent of the increase in incidence of primary cancer of the lung observed particularly among males in various countries and areas, or, where such increase is not found, the conditions of its absence.

3.1.2.—The association between the smoking of tobacco, especially cigarettes, and cancer of the lung.

3.1.3.—The atmospheric pollution in urban and industrial areas.

3.1.4.—The occupational or industrial exposure to hazards associated with an increased incidence of cancer of the lung.

3.1.5.—Environmental factors yet unknown including any that may act through ingestion, skin contact, etc.

3.1.6.—Variations in the susceptibility of individuals exposed to environmental factors causing cancer of the lung.

3.1.7.—Co-action of factors causing cancer of the lung.

3.1.8.—Histology of lung lesions caused by any of the categories of factors mentioned.

3.2.—That such studies be carried out as international cooperative and correlated studies in populations with high and low incidence of cancer of the lung with a simultaneous view to as many of the factors mentioned as possible.

ERRATUM

The following correction should be made in the abstract by B. Grad, A. Oberleitner, and J. Berenson ("The Effect of Thyroxine and Thiouracil on the Incidence of Lymphogenous Leukemia in Akr Mice") in the *Proc. Am. Assoc. Cancer Research*, 1:20, 1953. The last sentence of the second paragraph on page 21 reads: "Hence, the thyroxine mice were hyperthyroid, but nevertheless their survival was significantly lowered." It should read: ". . . was *not* significantly lowered."